THE HOST-PARASITE INTERACTION WITH PARTICULAR REFERENCE TO *TRYPANOSOMA EVANSI* ANTIGENS

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ABSTRACT OF THESIS

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The objective of the present study was to examine the ways that the *T. evansi* components interact with the host by investigating the components of the parasite which acted as antigens during infection and to study the dynamics of some of these antigens during infection. Results from this study could help in identifying new diagnostic reagents, a better understanding of existing diagnostic methods and target antigen for production of vaccines.

The antigenic components of intact and trypsin-treated *T. evansi* were identified. Thirty nine protein bands were detected in the trypanosomal preparations using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Twenty seven of these components were identified as antigens using immunoblotting against sera from infected rabbits and rabbits immunised with a range of soluble parasite materials. The solubilisation procedure and immunisation method affected the number of antigens recognised by these sera. The trypsin-treated parasites failed to produce any antibody response as measured by immunoblotting and ELISA.

Two soluble antigens identified by immunoblotting were selected for further study, a trypsin sensitive component of ~ 52 k.Da which was cleaved from the parasite by the process of trypsinisation and a non-surface component of ~ 42 k.Da which was recognised strongly by sera from infected rabbits and rabbits immunised with the parasite soluble materials. Both antigens were also immunogens as soluble extract. A third antigen of molecular weight of ~72 k.Da recognised by monoclonal antibodies raised to the intact living trypanosomes was also included in the study.

Polyclonal monospecific antibodies were produced to both the 52 k.Da and 42 k.Da antigens. The 72 k.Da monoclonal antibody and both polyclonal antibodies were labelled separately with biotin and horseradish peroxidase. These labelled antibodies were used to develop an enzyme-linked immunosorbent assay (antigen ELISA) to detect their corresponding antigens in the blood and tissue extracts of infected animals, and in immunohistochemical tests on cryostat sections to localise the antigens in the tissues.

The three antigens were detected in the blood of infected animals at different times. The 52 k.Da antigen was first detected in the blood 6 days after infection, followed by the 42 k.Da antigen 7 days post-infection and the 72 k.Da antigen 8 days after infection. Following the administration of Berenil, the 72 k.Da was cleared from the circulation the following day. The two other antigens were cleared from the circulation 2 days after treatment. In the tissue extracts, the 42 k.Da antigen was detected extravascularly from the spleen, brain and kidneys. In case of the 52 k.Da, it was also detected from the heart and lungs in addition to the above organs. The 72 k.Da antigen was, however, not detected in any of the tissue extracts. On cryostat sections prepared from the above organs however, none of the antigens was detectable.

The 52 k.Da antigen identified as an invariant antigen is a possible candidate for the diagnosis of infection but only in areas where *T. evansi* is the only trypanosome species present due to cross reaction.

The difference in the dynamics and localisation of the trypanosomes antigens warrants further investigation and provides a better understanding of the host-parasite interaction.

DECLARATION

This thesis has been composed by me and describes my own work

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DEDICATION

To *Hala* and *Mazin* for sacrifice without measure

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CHAPTER ONE

REVIEW OF LITERATURE

1. 1. INTRODUCTION:-

Trypanosomes are uniflagellated parasitic protozoa, the majority of which live and divide extracellularly in the blood and tissues of infected hosts.

Most of the parasites in the *Genus Trypanosoma* are of veterinary and medical importance causing widespread disease throughout many tropical and subtropical countries.

Trypanosoma evansi, the causative agent of the disease most commonly referred to as Surra was the first pathogenic trypanosome described (Evans, 1881-1882). Infection with *T. evansi* has an extremely wide geographical range in countries with hot and warm-temperate climates (Hoare, 1972) and infects a range of economically important livestock. Equids, camels, cattle, buffaloes, goats, sheep and pigs can all become infected (Luckins, 1988), but the principal affected hosts are equines, dromedaries and dogs (Hoare, 1972). A wide range of wild animals from wild rats to tigers and jaguars have been reported as naturally or experimentally susceptible to infection with *T. evansi* (Choudhury and Misra, 1972; Marinkelle, 1976; Sinha *et al*, 1971; Stephen, 1986). Usually *T. evansi* has a lethal effect upon most of these wild animals which are not regarded as sources of infection for domestic animals (Hoare, 1972). Successful transmission of *T. evansi* depends on factors such as vector preferences and closeness of donor to recipient host (Payne, 1988) which are not likely to be satisfied with these wild animals under normal livestock management practices. Hoare (1972) considered these wild animals as much victims of the infection as the livestock and they probably acquire the infection secondarily from the domestic animals.

The other major trypanosome antigens are invariant and common between stocks of the same species and between different species of trypanosomes (De Raadt, 1974, Burgess and Jerrells, 1985, Muller *at al*, 1992).

1. 2. 1. Variant surface antigens:-

Trypanosome infections are classically characterised by successive waves of parasitaemia each consisting of immunologically distinct parasite populations (Turner, 1982). This works in the parasites' favour as by the time that antibodies capable of clearing the circulation of the trypanosomes are produced by the host's immune system, other populations of parasites bearing different antigens on their surface emerge, evade the host's immune system and proliferate to continue the infection (Seed, 1972; Campbell and Phillips, 1976; Murray and Urquhart, 1977; Wellde *et al*, 1981). This cycle can continue, apparently indefinitely, usually leading to the death of the host if chemotherapy is not instituted (Gray, 1965; Turner, 1982; Donelson and Turner, 1985). Each antigenically distinct trypanosome population is commonly referred to as a variable antigen type (VAT). The variable antigen itself is a surface glycoprotein usually referred to as variant-surface glycoprotein (VSG) (Cross, 1975).

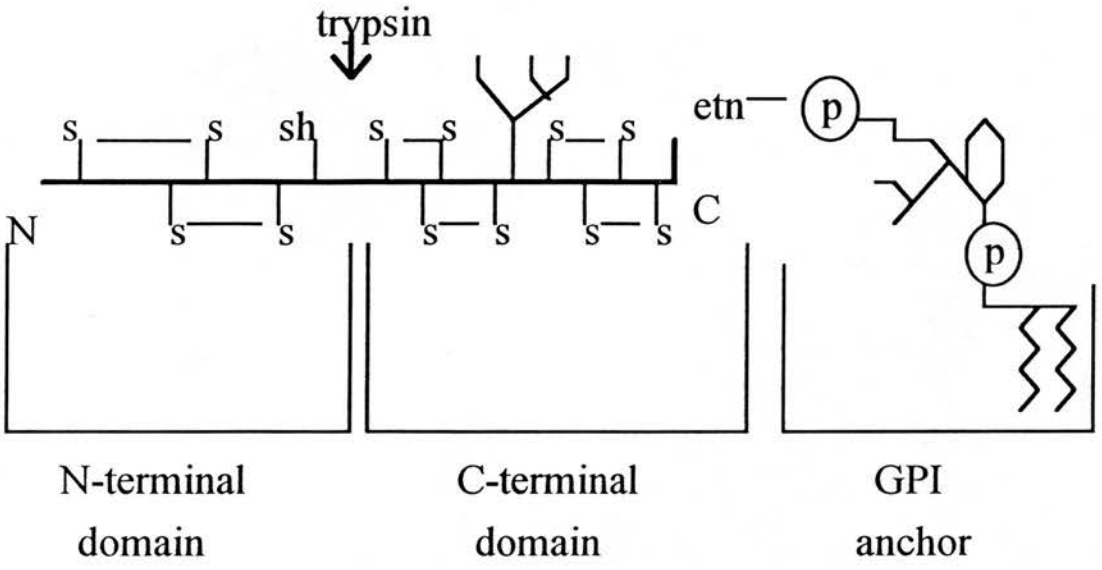
Ritz (1916) demonstrated that infections initiated with a single trypanosome can produce multiple variants. Although the total number of variant-surface antigens in the repertoire of a single infection is not known a single trypanosome of *Trypanosoma equiperdum* has been shown to give rise to over one hundred distinct antigenic types, with no indication that the repertoire had been exhausted (Capbern *et al*, 1977). Furthermore, genetic studies of *Trypanosoma brucei* VSG genes suggest that the genome has the potential to encode about one thousand VSG (Van der Ploeg *et al*, 1982).

The first indication that the variable antigen was located on the surface of the trypanosome came from the observation of Vickerman (1969) that the bloodstream and metacyclic stages of trypanosomes differed from other tsetse developmental stages in possessing a 12-15 nm thick surface coat. This coat corresponded to the variable antigen. and adapted the trypanosomes to life in a mammalian host by providing a replaceable surface which enabled the trypanosomes to evade immunological attack by the host. This role of surface coat in protection in the mammalian stages of the trypanosome life cycle has been confirmed and substantiated by subsequent workers (Vickerman and Luckins, 1969; Cross, 1975; Taylor and Cross, 1977; Fruit *et al*, 1977; Barry and Vickerman, 1979) who have demonstrated the presence of the variable antigen on the surface coat of the trypanosomes using a range of techniques.

Although chemically variable the surface coat of living trypanosomes is a stable structure and is attached to the cell membrane through a glycosyl-phosphatidylinositol (GPI) membrane anchor (Ferguson *et al*, 1988). GPI-anchored surface proteins are commonly found in other protozoa, in metazoa and in higher eukaryotes (Ferguson, 1994). GPI anchors act to insulate the intracellular components from external physicochemical attack as the GPI-anchored proteins do not possess cytoplasmic domains and therefore do not interact directly with intracellular components (Ferguson, 1994).

The trypanosome surface coat is susceptible to enzymatic digestion and is removed by the proteolytic enzyme trypsin (Wright and Hales, 1970; Cross, 1975; Frommel *et al*, 1988). Electron microscopy studies carried out by Cross (1975) showed that the surface coat could be uniformly removed from the trypanosomes by trypsin treatment without causing any other gross changes in morphology or affecting trypanosome motility. Trypsin only cleaves peptide bonds on the carboxyl side of arginine and lysine residues (Stryer, 1988). The

trypsin-sensitive region of the VSG molecule is the hinge region between the N- and C-terminal domains as shown in the following diagram adapted from Ferguson and Homans (1989).



Schematic representation of Variant Specific Surface Glycoprotein (VSG)

The susceptibility of different trypanosome species and variants to trypsin digestion is not uniform. In their study on the organisation of variant-specific surface antigens of *Trypanosoma brucei*, Cross and Johnson (1976) found that radiolabelled Concanavalin A (Con A) could bind to the trypanosome surface after brief trypsinisation. Under the conditions used, binding was optimal after 12 minutes trypsinisation, when the surface coat was no longer visible by electron microscopy. After 30 minutes, binding of Con A was negligible, trypanosome viability was poor and lysis was occurring. However, Frommel *et al* (1988) stressed that trypanosomes exposed to trypsin for 60 minutes were still motile and viable, and more than 90% of the variant-specific

glycoprotein was removed from the surface of all clones of *Trypanosoma brucei brucei*, *T. b. rhodesiense* and *T. b. gambiense* studied. This could be due to a difference in the accessibility of trypsin to the target amino acids and to differences in the density of the surface coat.

Removal of the surface coat of the trypanosomes by protease treatment was found to abolish the trypanosome infectivity (Cross, 1975). The exposure of the plasma membrane following removal of the surface coat by trypsin provides sites for activation of complement by the alternative pathway (Ferrante and Allison, 1983); when the trypanosomes are covered with C3b, they are phagocytosed by macrophages (Newton, 1981). The behaviour of trypsin-treated trypanosomes is similar to uncoated *T. congolense* culture forms trypanosomes and procyclic forms of *T. congolense* and *T. brucei* which are lysed by normal human serum while coated blood forms are resistant to serum lysis (Ferrante and Allison, 1983).

The molecular weight of VSG of most tsetse-transmitted trypanosome species ranges from 50 to 65 k.Da (Cross, 1975; Cross, 1977; Johnson and Cross, 1977; Hoeijmakers *et al*, 1980; Reinwald *et al*, 1978; Rovis *et al*, 1978; Richard *et al*, 1981; Ross *et al*, 1987). *T. vivax* has a reported VSG molecular weight between 46 and 50 k.Da (de Gee and Rovis, 1981; Gardiner *et al*, 1987). The VSG molecular weight of *T. evansi* clones studied ranged from 45 to 67 k.Da (Cross, 1979; Vervoort *et al*, 1981; Richards, 1984; Uche, 1989). There are thus very wide variations in the VSG molecular weight not only between different trypanosome species, but also between different stocks of the same species possibly due to different degrees of glycosylation of the different VSG molecules.

1. 2. 2. Invariant Surface Antigens:

Although the variant antigens of Salivarian trypanosomes constitute the major part of the surface, invariant surface antigens have also been reported. Infected mammalian hosts produce antibodies against minor and invariant surface antigens (Burgess and Jerrells, 1985; Frommel and Balber, 1987). These invariant surface antigens, reported mainly in *T. brucei*, are glycoprotein and referred to as invariant surface glycoproteins (ISGs); their abundance is said to be >100-fold lower than the VSG (Overath *et al*, 1994). These antigens are considered to be arranged in between the VSG molecules on the surface of the parasite. The 145 k.Da and 140 k.Da molecules are invariant surface glycoproteins which act as receptors for low-density lipoprotein (LDL) which is required for trypanosome growth (Hide *et al*, 1989; Coppens *et al*, 1988; Coppens *et al*, 1992; Rolin *et al*, 1990). Other ISG's function as glucose transporters (Parsons and Nielsen, 1990; Bringaud and Baltz, 1992). ISGs with as yet unknown function have been identified and include a 77 k.Da protein isolated from coated endocytotic vesicles of *Trypanosoma brucei* (Webster and Shapiro, 1990). Two invariant surface glycoproteins of 65 and 75 k.Da have been identified by Ziegelbauer and Overath (1992) in the bloodstream stage of *T. brucei* . These particular ISG's having large extracellular domains were initially thought to be the most abundant surface proteins with the exception of VSG (Overath *et al*, 1994). However, with the exception of a weak reaction with anti-ISG 75 antibodies, in live trypanosomes these ISGs were not considered to be accessible to antibodies (Ziegelbauer *et al*, 1992) and are not involved in protection against reinfection.

1. 2. 3. Non-surface antigens:-

The inherent variability of the trypanosome surface antigens and their poor potential for diagnosis of trypanosome infection has stimulated a search for

alternative parasite materials for diagnostic tests. However, little have been done on the role of internal trypanosome antigens in protection and control of the disease.

The way in which the host deals with the release of internal antigens after VSG-specific lysis is not fully examined, but could form an important part of the response to infection. Once the host has initiated immune-mediated parasite destruction, a range of non-surface parasite components will be exposed to the host. It is possible that the type of host response to such individual components might be important in influencing the final outcome of an infection. Evidence of the involvement of non-variant immunity comes from studies on *T. brucei* infected N›dama and Zebu cattle. Three internal antigens were found to play an important role in control of the infection by these animals (Shapiro and Murray, 1982). These antigens, with molecular weights of 300, 150 and 110 k.Da, were recognised more frequently than other antigens. Animals with the capacity to recognise one or more of these antigens appeared to control parasitaemia better than those which did not recognise these antigens. Two antigens, a 69 k.Da and a 33 k.Da proteins from *T. congolense* have been associated with increased resistance to infection and elicited an IgG1 response in N›dama but not in Zebu cattle (Authie *et al*, 1993). Uche (1989) studied antibody response to a number of non-surface antigens of *T. evansi*. He showed that the number of trypanosome antigens recognised by IgG antibodies in rabbits during the period of primary infection ranged from 16 to 25 components and were dominated by the response to six major proteins with molecular weights of 94, 85, 75.5, 43, 32.4 and 28.6 k.Da. Animals that responded to a high proportion of these non-surface antigens during early infection were found to be more resistant to infection than those which responded to fewer antigenic components of the

parasite. He concluded that the immune response to non-surface antigens could be important in determining the outcome of *T. evansi* infection.

The internal components of the trypanosomes are estimated to constitute about 90% of the total protein of the bloodstream forms of *T. brucei*, with the remaining 10% attributed to the surface coat (Cross, 1990). Roelants and Pinder (1984) suggested that the internal antigens of trypanosomes could play an important role in the pathogenesis of the disease not only by triggering an immune response after the destruction of the parasite by VSG specific antibodies but also by the consequential formation of immune complexes . The deposition in the kidneys of immune complexes formed by the interaction of internal trypanosome antigens and antibodies, was found to be responsible for nephritis in infected animals (Boreham and Kimber, 1970; Nagle *et al*, 1974). Free internal antigens were also found to contribute to the pathology of the disease. The highly antigenic enzyme cysteine proteinase of *T. cruzi* has been found to contribute to the pathology of the disease by degrading proteins across a wide range of pH's and by stimulating immune T cells from chronic chagasic patients resulting in inflammation (Murta *et al*, 1990).

Many of the non-surface antigens of trypanosomes are non-variable and stable components across trypanosomes species (De Raadt, 1974). This stability makes them better prospects as antigens for diagnostic tests than the parasite VSG. Two *T. brucei* non-variable antigens recognised early during infection by different mammalian hosts have been identified and characterised as microtubule proteins, designated GM6 and MARP1, (Muller *et al*, 1992). Both antigens were present in bloodstream and procyclic forms of the parasite. Moreover probing whole cell extracts of *T. evansi*, *T. congolense*, *T. rangeli* and *T. cruzi* with hyperimmune sera raised against these antigens indicated their presence in all these parasites. This makes them candidates for

immunodiagnosis of trypanosomal infections at an early stage irrespective of trypanosome species or serodeme particularly in areas where only one species of trypanosomes is present.

1. 3. EXTRAVASCULAR TRYPANOSOMES:-

Trypanosomes of the subgenus *Trypanozoon* are known to inhabit both vascular and extravascular sites throughout the host (Seed and Effron, 1973; Seed *et al*, 1984; Tanner *et al*, 1980; Turner *et al*, 1986; Sudarto *et al*, 1990). These extravascular trypanosomes were suggested as the source of relapse populations following remission of the parasitaemic wave (Jennings *et al*, 1979). Their presence in tissues also contribute to the pathology of infection due to local tissue damage (Losos and Ikede, 1972). The presence of degenerated trypanosomes in the internal organs of infected host (Ormerod and Venkatesan, 1971a; Ormerod and Venkatesan, 1971b, Schmidt and Bafort, 1987) and the sequential release of the parasite antigens in these tissue may, however, play an important role in the defence against the infection by forming a continuous source of stimulation of the immune system.

1. 4. DETECTION OF TRYPANOSOME ANTIGENS:-

Most of the diagnostic assays are directed at detecting trypanosomal antibodies and are dependent on the availability of specific parasite antigens. Antigen-trapping assays for the diagnosis of trypanosomal infections have recently been developed and are dependent on the availability of specific antibodies. Two types of materials can be used in these assays, blood which

contains whole trypanosomes, products of lysis, degenerating trypanosomes and immune complexes. Tissues containing extravascular trypanosomes, degenerating parasites and depots of immune complexes.

1. 4. 1. Sources Of Antibodies:-

Different types of antibody sources can be utilised for identification and detection of parasite antigens.

Trypanosomes, being extracellular blood parasites provoke primarily an antibody response (Urquhart and Holmes, 1987). Infected animals produce antibodies against both surface and non-surface antigens (Musoke *et al*, 1981; Shapiro and Murray, 1982; Uche, 1989). This type of antiserum is a useful indicator of the parasite antigens that are exposed to the animal during infection. Antibodies in this type of serum are, however polyspecific, with different levels of affinity and do not discriminate between individual trypanosome antigens. Another disadvantage of infection serum is that the response to trypanosome infection varies not only between different species of animals but also between animals of the same breed. Some animals respond to a large number of trypanosome components while others respond to only a few components (Shapiro and Murray, 1982, Uche, 1989).

Hyperimmune serum to crude extracts of trypanosomes is another type of antibody source for use in immunoassays. (Tijssen, 1985). This type of sera often contains high levels of high affinity antibodies (Harlow and Lane, 1988). Hyperimmune serum contains polyspecific antibodies that do not discriminate between individual antigens. Moreover, immunising with crude extracts of trypanosomes may expose parasite components that are not usually antigenic during the course of infection and do not reflect the parasite components that are accessible to the immune system during infection.

Hyperimmune serum to single parasite antigens is a third type of antibody source for use in immunoassays. Antibodies in this type of serum contain high proportions of high affinity antibodies. The monospecificity of these antibodies makes them better reagents for use in immunoassays than those raised to crude trypanosomes extracts. The high affinity and high detectability of these polyclonal monospecific antibodies also makes them better reagents in immunoassays compared to monoclonal antibodies (Tijssen, 1985). However, there are a number of factors that determine a successful antibody response to single antigens, these include the nature of the antigen, the degree of purity, its relative immunogenicity, its physical form and the method and amount of administration (Tijssen, 1985). Large molecules such as proteins and polysaccharides are strongly immunogenic, while lipids, steroids and nucleic acids are weak immunogens (Barriga, 1981, Tijssen, 1985). A high degree of purity of the antigen is important when highly specific antiserum is required as in case of immunoassays since the sensitivity of these assays is dependent on the specific activity of the antibody. Small amounts of an immunodominant contaminant in the antigen sample may overwhelm the response to the principal antigen due to antigenic competition (Tijssen, 1985). The physical form of the antigen is also known to affect the host response; soluble antigens in their native form are usually poor immunogens while aggregated, polymeric, antigens are good immunogens (Dresser, 1962).

The fourth type of antibody source for immunoassays is the production of monoclonal antibodies. Monoclonal antibodies are highly selective reagents that can be used to identify individual antigens in complex mixtures such as trypanosomes. Unlike polyclonal mono-specific antibodies, a pure antigen is not required for immunisation of animals for monoclonal antibody production. Another advantage of this type of antibody over the polyclonal sera is that the

hybridoma cells secreting the desired antibody can produce unlimited quantities of antibodies with defined specificity thus representing a continuous source of antibody. A disadvantage of the monoclonal antibodies is their lower detectability in immunoassays compared to polyclonal antibodies due to their low avidity and the small fraction of high-affinity antibodies among them (Tijssen, 1985).

1. 4. 2. Separation and purification of antigens:-

Trypanosomes are easily separated from host blood cells by ion-exchange chromatography (Lanham and Godfrey, 1970) using DE52 cellulose. Extracts of trypanosomes, for production of antibodies to different components of the parasite or for identification of the parasite antigens, are usually obtained by disruption of the parasites by treatment with ultrasound (Brown and Williamson, 1962), by freezing and thawing (Hughes, 1951; Weitz, 1960; Johnson *et al*, 1963) or by ionic and non-ionic detergent lysis (Cardoso de Almeida and Turner, 1983, Frommel *et al*, 1988). Protease inhibitors are usually included in the solublisation medium to block the activation of the endogenous proteases present in the trypanosomes such as lysosomal cysteine proteinases, cytosolic alkaline peptidases, acidic proteinases, amino-peptidases and metalloproteinases (North *et al*, 1983, Lonsdale-Eccles and Mpimbaza, 1986, Lonsdale-Eccles and Grant, 1987) released by these solublisation methods.

Trypanosomes consist of a complex mixture of chemical components, and purification of individual trypanosome antigens from such materials is usually based on their physicochemical properties. Most of the trypanosomes antigens described so far are either proteins (Shapiro and Murray, 1982, Muller *et al*, 1992, Authie *et al*, 1993) or glycoproteins (Cross, 1975, Frommel and Balber, 1987). Most of the work carried out on the purification of trypanosome antigens has been directed to the variant specific glycoprotein. This is possibly

due to its high degree of immunogenicity, its role in protecting animals against reinfection (Wellde *et al*, 1981, Hall and Esser, 1984) its abundance (it constitutes 10% of the total trypanosome materials. Cross, 1990) and the relative ease with which it can be separated from other trypanosome materials. Purification methods used to purify the VSG molecules involve chromatographic separation including high performance ion-exchange chromatography (Clarke *et al*, 1984) using a packed column of particulate material consisting of a rigid, polymeric substrate to which charged groups are bound (fixed charges). Proteins and peptides (mobile ions) will attach to the fixed charges via ionic interactions. The fractionation is then achieved by altering the pH or increasing the ionic strength of the medium (Henry, 1989). Size exclusion chromatography has also been used to purify the VSG (Down *et al*, 1991). In this method, molecules in solution are separated according to differences in their molecular sizes as they pass through a column packed with a chromatographic medium which is a gel. Large molecules are usually separated first followed by smaller ones whose passage is delayed by their diffusion into and out of the gel matrix. Affinity chromatography using lectins (Johnson and Cross, 1977, Strickler *et al*, 1978, Reinwald *et al*, 1979) or antibodies (Pearson and Anderson, 1980) have also been used in purification methods, however, different VSG molecules have different affinity for lectins (Johnson and Cross, 1977; Turner, 1982) and antibody-affinity chromatography requires specific antisera or monoclonal antibodies.

The number and heterogeneity of the non-variant protein antigens of trypanosomes necessitates the application of different purification methods both separately and in combination. A relatively simple method for the separation and purification of protein antigens is by sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE) (Harlow and Lane, 1988).

Although this method involves denaturation and may lead to changes in epitope conformation, SDS-denatured antigens usually retain their antigenicity and elicit strong antibody responses (Lazarides and Weber, 1974, Stumph *et al*, 1974). This method of purification of antigens has been used to analyse the response to antigens from a wide range of organisms (Strauss *et al*, 1975, Carrol *et al*, 1978, Boulard and Lecroisey, 1982). An important part of using SDS-PAGE purification of antigens is the efficient localisation of the target antigen in the gel therefore avoiding extensive fixation and denaturation of the antigen that can be caused by the staining procedure. Localisation methods include the use of a stained reference lane to localise the band of interest in the rest of the gel. Misalignment of the reference lane and the rest of the gel can, however, result in contamination of the chosen antigen Alternatively the whole gel can be stained with a light stain such as Coomassie blue, sodium acetate or copper chloride to locate the target antigen. This method permits the separation of a single protein band with a minimum risk of contamination by nearby protein bands. The excised gel slice containing the target antigen can then be fragmented by repeated passage through a syringe and injected into animals for production of antibodies (Strauss *et al*, 1975, Boulard and Lecroisey, 1982). Fragmentation, however, depends on the concentration of acrylamide and thickness of the gel as thick gels are difficult to fragment. Alternatively, proteins trapped in polyacrylamide gel can be electro-eluted from the polyacrylamide and used for immunisations (Harlow and Lane, 1988). An advantage of this method is that, the concentration of the eluted protein can be measured and the antigen can be incorporated with an adjuvant before injection into animals.

1. 4. 3. Identification of antigenic components of trypanosomes:-

The development of SDS-PAGE, (Laemmli, 1970) provided a powerful method of resolving and analysing protein mixtures such as trypanosomal

material. Shapiro and Murray used SDS-PAGE in combination with immuno-precipitation to assess the humoral response to *T. brucei* infection in cattle. The fractionation of proteins by SDS-PAGE is based on the molecular size of the polypeptide chain of each component. The treatment of the parasite materials with an ionic detergent such as SDS will lead to the binding of large amounts of this highly charged detergent molecule (Reynolds and Tanford, 1970) which is sufficient to effectively overwhelm the intrinsic charges on the polypeptide chain so that the net charge per unit mass becomes approximately constant. The migration of proteins is therefore proportional to their molecular weight. The separated constituents can be visualised easily with protein stains such as Coomassie Blue. The antigenic identification of components with specific antibodies, however, is not easy to perform directly on the polyacrylamide gel, since macromolecules buried within the gel matrix have different accessibility to reagents. This is particularly important when gradient gels are used, since different parts of the gel have different porosities (Andrews, 1988). These problems are overcome by transfering the separated proteins to a solid matrix such as nitrocellulose (NC) to which they bind and are immobilised before they can be probed for antigenicity. The process of transferring separated proteins and nucleic acids from gels to a solid matrix is referred to as "blotting". The most widely used transfer method is the one employing electrophoretic transfer of proteins to NC which is commonly referred to as "western blotting" (Towbin *et al*, 1979; Burnette, 1981) or electroblotting.

There are several different types of immobilising matrix used for blotting such as diazobenzyloxymethyl paper(DBM), diazophenylthioether (DPT), nylon-based membranes and NC. The activated papers (diazo groups) covalently bind proteins and have good mechanical strength, but the coupling method is incompatible with many gel electrophoresis systems since the linkage is through

primary amine groupings. They are also expensive and the reactive groups have a limited half-life. Nylon has excellent mechanical strength and does not shrink by washing or drying, but it can only bind to a small amount of protein and is therefore not suitable for most applications (Harlow and Lane, 1988). The most commonly used material is NC which has a protein binding capacity of 80 - 100 $\mu g/cm^2$. Although the mechanism of protein binding to NC is not well understood, it is thought to involve hydrophobic interactions (Andrews, 1988).

Western blotting has been used extensively for the study of parasite systems including several species of trypanosomes. Cardoso de Almeida and Turner (1983) used the technique to investigate the properties of the membrane form variant surface glycoprotein of *T. brucei*, while Gardiner *et al* (1987) used it for the identification and isolation of *T. vivax* VSG. The technique was also used to determine the molecular nature of invariant antigens in *T. brucei rhodesiense* (Burgess and Jerrells, 1985) and the presence of glycoproteins in *T. brucei* using lectin blotting (Frommel and Balber, 1987). Uche (1989) used the technique to study antibody responses to *T. evansi* antigens in experimentally infected rabbits.

1. 4. 4. Assay for antigens in the host:-

Soluble trypanosome antigens circulating in body fluids of infected animals have been detected using enzyme linked immunosorbent assay, ELISA, (Rae and Luckins, 1984; Nantulya *et al*, 1989, Olaho-Mukani *et al*, 1989; Masake and Nantulya, 1991, El Amin *et al*, 1993, Olaho-Mukani *et al*, 1993, Sinyangwe and Munyama, 1993). The assay offers many advantages over other immunoassays in terms of speed, sensitivity, convenience, simplicity and reliability. The test is based essentially on two fundamental steps, the

immunological reaction between the antibody and antigen and the enzymatic indicator reaction to demonstrate the presence or absence of antibody-antigen reactions (Kurstak, 1985).

Competitive and non-competitive assays can be used to detect the soluble antigens in the test sample. Competitive assays are based on the competition of the test antigen with a standard amount of enzyme-labelled antigen for the immobilised antibody (WHO, 1976). The decrease in staining indicates the presence of antigen in the test sample. The non-competitive assay or double antibody sandwich method is the most popular method, however, it depends on the antigen having at least two epitopes (Tijssen, 1985). In this method the antibody is immobilised on the solid matrix followed by incubation with the test sample containing the antigen and the reaction is then detected by the addition of the primary antibody labelled to an enzyme. The detectability of this method for the detection of antigen or antibody can be improved by use of bridge methods, in which the captured antigen or antibody is detected by nonimmunologic methods such as linked avidin-biotin system (LAB) (Kurstak, 1985). In this modification the bound antigen or antibody is reacted with a biotinylated secondary antibody directed against the bound molecule and the complex is then detected by the addition of avidin-enzyme conjugate (Figure 1.1).

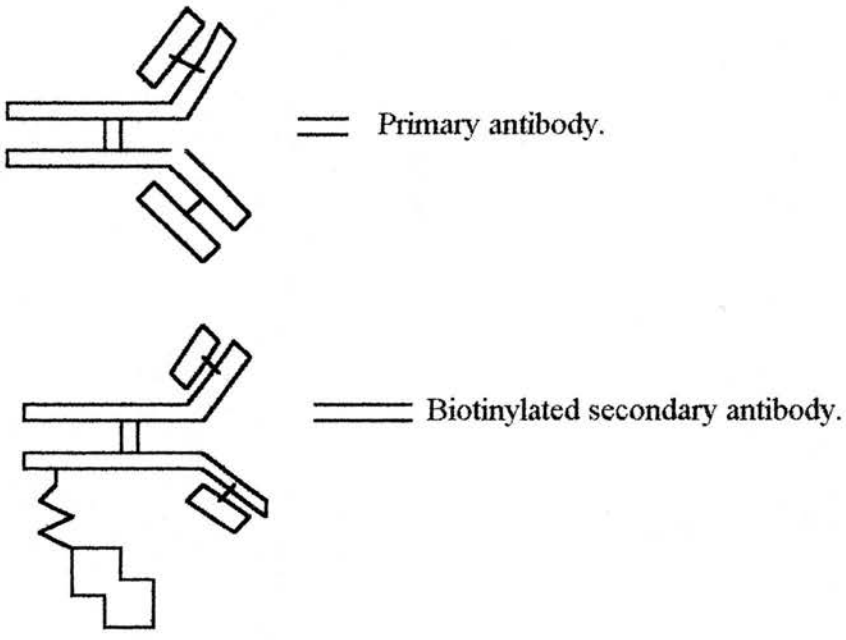
Of the factors influencing the performance of solid-phase enzyme immunoassays, the solid matrix, the quality of antibodies and antibody-conjugate are the most important (Tijssen, 1985, Kemeny, 1992). The choice of the solid matrix or carrier depends on the purity of the coating material, the presence or absence of non-specific binding and the sample concentrations which have to be assayed (Kemeny, 1992). Two types of carriers can be used based on their capacity to bind proteins. High capacity carriers such as cellulose

Figure 1.1.

The Linked Avidin-biotin system (LAB) in enzyme immunoassays

N.B.

Ag = antigen
A = avidin.
E = enzyme.



AG
A
Substrate
AG
A
Substrate

and nitrocellulose are stable and can be stored for long periods after being coated. They are particularly suitable for impure coating materials such as crude extracts. However, they are difficult to wash and give higher background binding compared to low capacity carriers. Low capacity carriers such as plastic are most commonly used in ELISA, they are easy to wash and produce low background binding (Kemeny, 1992). 96-well polystyrene microtitre plates (Voller and Bidwell, 1975) are the most commonly used plastic carriers in ELISA. They are easy to handle and multiple samples can be tested per plate. High affinity antibodies are usually required in immunoassays because they bind large amounts of antigen and consequently produce a strong signal in a short period of time (Harlow and Lane, 1988). Low affinity-antibodies bind only a small proportion of the antigen and therefore require a proportionately longer period of time to develop a detectable signal. The affinity of the antibody also has an effect on the avidity of the antigen-antibody complex because antibodies with a high affinity increase the stability of the antigen-antibody complex during washing steps (Tijssen, 1985), therefore increasing the final signal strength. As in the case of unlabelled antibody, the specificity of the labelled preparation is also important.

The development of immunohistochemical techniques has facilitated the identification and localisation of the antigens in the tissues. Immunofluorescence as a method of detecting antigens in tissues has increasingly been replaced by enzyme immunoassays (Tijssen, 1985). The properties of the enzymes made much more sensitive modifications possible. such as the peroxidase-anti-peroxidase (PAP) method and avidin-biotin-peroxidase complex. Enzyme immunoassays have been successfully applied for the detection and localisation of antigens in cells or tissues (Naritoku and Taylor, 1982, Mwangi *et al*, 1990, Sudarto, *et al*, 1990). Paraffin sections from

fixed tissues or cryostat sections of frozen, fresh or fixed tissues are usually used in such tests (Tijssen, 1985, Harlow and lane, 1988). Although both sections have been successfully used, paraffin sections did not preserve many antigens (Tijssen, 1985). While cryostat sections are reputed to preserve antigens and cell structures (Harlow and Lane, 1988) the diffusion of soluble antigens will decrease their detectability. The tissues are usually fixed to avoid structural decomposition and to reduce the diffusion of soluble antigens. However, depending on their chemical composition antigens may be extracted or denatured by fixative agents such as methanol or acetone which are suitable for large proteins (Tijssen, 1985).

1. 5. ANTIGEN DYNAMICS DURING INFECTION:-

Typically the recognition of any antigenic substance, either free in solution or constituting part of a more complex structure begins with its phagocytosis and processing by antigen-presenting cells (Barriga, 1981). In trypanosomes, however, phagocytosis is antibody-dependent as revealed by both *in vitro* and *in vivo* studies. *T. brucei* and *T. gambiense* were found to attach and be ingested by macrophages *in vitro* in the presence of immune serum (Lumsden and Herbert, 1967; Takayanagi *et al*, 1987). MacAskill *et al* (1980) evaluated the process of phagocytosis in the absence of antibody and found that, suppression of antibody production by prior irradiation, at a level which did not impair phagocytic function, abolished the ability of infected and treated mice to remove high levels of radiolabelled *T. brucei*. In *vivo* studies using ^{75}Se-labelled *T. brucei* also showed that, while labelled parasites remained in the blood of normal mice, they were rapidly removed from the circulation of immune mice mainly through hepatic uptake (Holmes *et al*, 1979). Moreover the antigen-presenting cells particularly macrophages have been shown to play no role in the

response to trypanosomiasis. Paulnock *et al* (1988) observed a significant reduction in the proliferative response by T cells when macrophages from *T. rhodesiense* infected mice were used as antigen-presenting cells, although these cells were capable of processing and presenting heterologous antigen.

The initial response in trypanosome-infected animals involves IgM antibodies specific to variant surface antigens (VSG), this response is later dominated by an IgG response. The protective immunity to trypanosomes is mainly antibody-mediated rather than cell-mediated immunity. Protection against homologous *T. brucei* infection in mice can be achieved through transfer of immune serum or by transfer of B lymphocyte enriched fractions of spleen cells (Takayanagi and Enriquez, 1973; Takayanagi and Nakatake, 1975; Campbell and Phillips, 1976; Seed, 1977). A strong correlation between protection and the level of neutralising antibodies has been demonstrated in cattle immunised with irradiated whole *T. brucei* trypanosomes (Morrison *et al*, 1982) or with *T. brucei* purified VSG (Wells *et al*, 1982). In *vitro* studies showed that VSG-specific antibodies are capable of causing various forms of damage to trypanosomes including trypanolysis (Shakibaei and Frevert, 1992), decrease in respiratory rate (Desowitz, 1956), agglutination (Cunningham and Vickerman, 1962; Takayanagi *et al*, 1991a; Takayanagi *et al*, 1991b; Takayanagi *et al*, 1992a; Takayanagi *et al*, 1992b), and opsonisation (Lumsden and Herbert, 1967).

Most of the information on the host-parasite interaction is based only on the behaviour of the whole parasites. The dynamic aspects and the fate of individual trypanosome components during infection are, however, not well understood. Information on these aspects might provide better insight into host-parasite interaction acting as indicators of infection, progress of the disease and effectiveness of chemotherapy. Most of the information on the dynamics of

trypanosome antigens during infection has come from studies designed to produce diagnostic tests based on antigen detection. In developing an antigen-ELISA for the diagnosis of bovine trypanosomiasis using monoclonal antibodies specific to the plasma membrane antigen of procyclic trypanosomes, Nantulya and Lindqvist (1989) reported variations in the antigen dynamics of different trypanosomes species. *T. vivax* and *T. congolense* antigens were detected as early as 10 to 12 days following tsetse challenge, while *T. brucei* antigens were detectable between 8 and 14 days post-infection. Following treatment, *T. vivax* and *T. congolense* antigens disappeared from circulation within two weeks but the rate of *T. brucei* antigens was slower compared to that of the other two species. The appearance of these antigens and their rate of clearance from blood is also influenced by the species of infected host. Using the above *brucei*-group-specific monoclonal antibody El Amin *et al* (1993) in evaluating antigen-ELISA for the diagnosis of *T. evansi* infections in camels and goats detected the antigen as early as 2 - 4 days post-infection in camels. In goats the antigenaemia was detectable 7 days after infection. Following treatment of camels with Trypacide the antigenaemia dropped within two weeks of treatment until disappeared from circulation 35 days later.

Investigations in this study were aimed at the identification and antigenic characterisation of individual components of *T.evansi* which acted as antigens during infection. The dynamics of some of these antigens was studied during the course of infections in mice and rats.

CHAPTER TWO

GENERAL MATERIALS AND METHODS

This chapter includes methods used in more than one area of the study.

2. 1. TRYPANOSOMES

Two stocks of *Trypanosoma evansi* originally isolated from naturally infected buffaloes from Indonesia were used - one population of BAKIT 374 and two variants of BAKIT 259A. The passage and isolation history of these populations is presented in Figure 2. 1.

2.2 EXPERIMENTAL ANIMALS

2. 2. 1. Mice:

The mice used for expansion of trypanosomes were female random bred albino white mice "TO" (A. Tuck & sons, England) weighing 20-30 g.

2. 2. 2. Rabbits:

The rabbits used for the production of infection serum and polyclonal antibodies against *T. evansi* materials were female adult New Zealand White rabbits (Hyline Commercial Rabbits, England) weighing between 2 - 2.5 Kg.

2. 3. SEPARATION OF TRYPANOSOMES FROM HOST BLOOD:

Cryopreserved trypanosomes were grown in mice until fulminating infection developed. The mice were exsanguinated by cardiac puncture under general anaesthesia and blood collected into a 1 ml syringe containing 10 i.u. of

heparin in 0.1 ml of phosphate buffered saline (PBS) (Appendix 1). Trypanosomes were then separated from host blood cells by ion-exchange chromatography on DEAE cellulose (DE52, Whatman Biochemical UK.) as described by Lanham and Godfrey (1970). The separated trypanosomes were washed three times in phosphate buffered saline glucose (PSG) pH 8.0 (Appendix 2) by centrifugation at 2650g for 20 minutes at 4°C.

2. 4. ESTIMATION OF TRYPANOSOMES CONCENTRATION:.

The concentration of the trypanosomes used for infection was determined by counting the parasites in a haemocytometer slide (Neubauer, Improved Double Ruling Weber, UK). Five µl of a trypanosome suspension diluted to 1/1000 in PSG was carefully transferred to the haemocytometer slide by touching a pipette tip onto the coverslip edge. Using a microscope (x 400 magnification, Leitz Wetzlar Laber-Lux, Germany) the number of trypanosomes in 16 small squares (one large square) were counted. All trypanosomes touching the left and upper borders were included. The total number of trypanosomes in the four large corner squares was determined and the mean calculated. The number of trypanosomes per ml was determined using the following formula:Trypanosomes / ml = mean no. of trypanosomes in the 4 squares x 10^4 x dilution factor

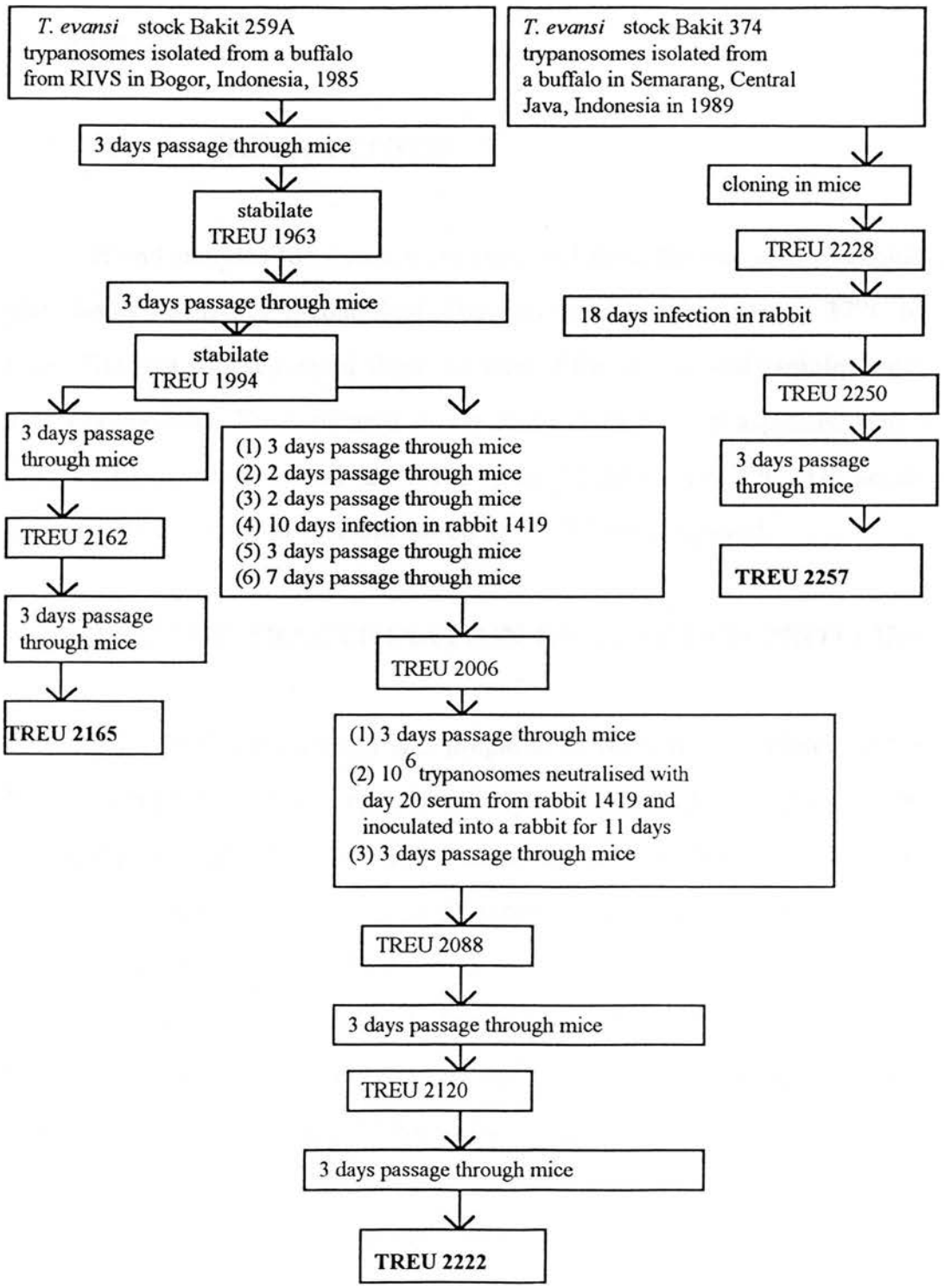
2. 5. ROUTINE MONITORING FOR TRYPANOSOMES:

Tail blood from mice or blood from rabbit ear vein was examined to monitor the progress of infection in infected animals. The blood was examined microscopically (20 microscopic fields at x 400 magnification) either as a wet blood smear on a slide (Baker, 1970) or using the microhaematocrit

centrifugation technique (MHCT) (Woo, 1970). For the MHCT the heparinised glass capillary tubes containing the centrifuged blood were cut at the buffy coat area using a diamond pencil. The cut ends of the capillary tube were then touched on a microscope slide to expel the contents of the buffy coat as well as the plasma and erythrocyte areas adjacent to the buffy coat.

Figure 2. 1

The history of *T. evansi* trypanosomes stocks



conducted in a vertical gel tank apparatus containing electrode buffer, pH 8.3 (25 mM tris, 0.192 M glycine, 0.1% SDS, Appendix 4) in both top and bottom reservoirs. 50 µl of sample per well and one well of 20 µl of proteins of known molecular weights (Low molecular weight markers , Pharmacia) were loaded onto each gel. The sample separation was carried out at room temperature, initially with a constant voltage of 150V for one hour, then at constant 100V overnight. After electrophoresis gels were removed for protein detection from the glass plates and stained with Coomassie Brilliant Blue R-250 (Sigma Ltd., UK.) by immersing the gels in 0.1%(w/v) Coomassie Brilliant Blue in destain solution (Appendix 5) at room temperature with gentle shaking for 60 minutes. The gels were then destained with destain solution up to 2 hours or until the background cleared. A calibration curve based on the molecular weight markers was constructed and used to calculate the molecular weight of individual trypanosome proteins. For permanent records the gels were rinsed in distilled water and photographed on a Polaroid film (Type 55; 4x5 inches; Sigma Ltd. UK.) with Polaroid camera (MP.4 Land camera) using a 35 Kodak Wratten gelatin filter.

2. 7. 1. Electrophoretic transfer of *T. evansi* proteins to nitrocellulose membrane:

The unstained gel containing *T. evansi* proteins separated by SDS-PAGE was soaked in transfer buffer (Appendix 6) and laid on two pieces of filter paper (6 µm pore size, Whatman) pre-soaked in the transfer buffer and laid on top of the anode plate of a Sartoblot^{R} II-S semi-dry electroblotter (Sartorius Ltd., Germany). A nitrocellulose membrane (0.45 µm pore size, Sartorius Ltd., Germany) previously cut to the size of the gel and soaked in transfer buffer was then laid on top of the gel and again covered by two soaked filter paper at the

cathode side. Electrophoretic transfer to nitrocellulose membrane was conducted for 90 minutes at room temperature with a constant current of 0.8 mA per cm^2 of gel.

Detection of the antigenic components of *T. evansi* on nitrocellulose membrane:

Following the transfer of *T. evansi* proteins onto nitrocellulose membrane, the membrane was transferred into a sandwich box and the excess protein binding sites blocked in 5% dried milk (Marvel, Premier beverages, Stafford, UK) in blocking buffer, pH 7.4 (Appendix 7) overnight with gentle shaking. The blocked membrane was rinsed with PBS and incubated overnight with the appropriate dilution of *T. evansi* antibodies in blocking buffer at room temperature. The unbound antibodies were then removed by washing the membrane with 7 changes of PBS over a period of 2 hours, before a peroxidase-labelled, donkey anti-rabbit IgG (Scottish Antibody Production Unit, Carluke, Lanarkshire) diluted to 1/500 in blocking buffer or goat anti-mouse IgG (Pierce, USA) diluted to 1/2500 was added and incubated for 2 hours. After washing the membrane in PBS as above to remove excess conjugates, the labelled antigen/antibody complexes were visualised by incubation in 4-chloro-1-α-naphthol substrate solution, pH 7.5 (Sigma Ltd., UK.) (Appendix 8). Colour development was allowed to occur over 20 - 30 minutes, and the reaction was stopped by washing in distilled water and permanent records made by photography.

2. 7. 2. Elution of proteins from polyacrylamide gels:

T. evansi proteins were eluted from Coomassie stained SDS-PAGE gels using an electro-eluter (Model 422 Electro-Eluter, Bio-Rad, USA) according to

the manufacturers instructions. Dialysis membrane caps, molecular weight cut off of 3,500 daltons, were soaked at 60°C in elution buffer (Appendix 4) for one hour. Glass tubes with frits in their bottom were placed into the electro-eluter module. The pre-wetted membrane caps were placed in bottom of silicone adapters. The adapters were then filled with elution buffer and slid onto the bottom of the glass tubes. Each tube was then filled with elution buffer and the gel slice containing an appropriate protein component was placed in each tube. The entire module was then placed in the buffer chamber of the equipment which was then filled with elution buffer, the level of which covers the silicone adapters. The upper buffer chamber was also filled with elution buffer and a stir bar was placed in the bottom buffer tank to prevent bubbles from sticking to the bottom of the dialysis membranes. Elution was performed at 10 mA per glass tube for 5 hours. For recovery of the eluted protein, the glass tubes were removed from the apparatus and the elution buffer in the tubes removed to the level of the frit using a plastic pipette and the liquid was discarded. The silicone adaptor together with the membrane cap containing approximately 400 µl of the eluted protein were then removed from the bottom of the glass tube and the protein was collected into a microcentrifuge tube using a plastic pipette. The membrane was rinsed with a further 200 µl of fresh elution buffer and added to the content in the microcentrifuge tube and stored at -20°C until needed.

2. 8. ENZYME-LINKED IMMUNOSORBENT ASSAY (ANTIBODY-ELISA) FOR *T. EVANSI* ANTIBODIES:

This assay was used to monitor *T. evansi* antibodies in rabbits and to test the reaction with monoclonal antibodies to the soluble materials of the parasite. ELISA microtitre plates (Immulon 1, Dynatech, USA) were coated with *T.*

evansi soluble materials diluted in carbonate / bicarbonate buffer, pH 9.6 (ELISA coating buffer, Appendix 9). 100 µl of the diluted *T. evansi* antigen was dispensed into each well and the plates were then incubated overnight at 4°C. Unadsorbed antigen was washed out of the wells with 3 x 1 minute washing cycles with PBS/Tween, pH 7.4 (Appendix 10) using an automatic ELISA washing machine (Dynatech). Antibody samples were diluted in PBS/Tween to the required range and 100 µl aliquots dispensed into the appropriate wells of the antigen coated plates. The plates were incubated at 37°C in a Varishaker-Incubator (Dynatech) for 30 minutes and the unbound antibodies removed by washing in PBS/Tween as described above. A peroxidase-labelled donkey anti-rabbit IgG (Scottish Antibody Production Unit) or goat anti-mouse IgG (Pierce, USA) was diluted in PBS/Tween to the appropriate concentration and 100 µl aliquots dispensed into each well of the microtitre plates. The plates were incubated at 37°C for 30 minutes and the unbound conjugate removed by washing in PBS/Tween as above. The enzyme labelled immunoglobulin complexes were then visualised by adding 100 µl / well of 3,3',5,5'-Tetramethylbenzidine dihydrochloride (TMB substrate, Sigma Ltd. UK.) in 0.05 M phosphate-citrate buffer, pH 5.0, containing 0.03% (w/v) sodium perborate and the plates incubated at 37°C for 15 minutes. The reaction was stopped by adding 50 µl 2M sulphuric acid to each well and the absorbance of each well was then measured photometrically at 450 nm using an ELISA plate reading photometer (Multiskan^{R} Plus, Labsystems, Finland).

2. 9. AGGLUTINATION TEST:

This test was used to examine the agglutinating ability of the *T. evansi* polyclonal monospecific antibodies and monoclonal antibodies. Terasaki pattern

microplates (Nunc HLA plates, 60x10 PS SH, Inter Med, Denmark) were flooded with approximately 5 ml of mineral oil (light white oil, Sigma chemical company, St. Louis, USA). A 2-fold dilution range from 1/2 to 1/1024 of serum in borate buffer, pH 8.0 (Appendix 11) were made in a 96-well microtitre plate and 2 μl were dispensed into the appropriate wells of the Terasaki microplates. 2 μl of borate buffer were also added to one of the wells as negative control, and the plates were incubated overnight at 4°C. Using a repeating dispenser, PB-600-1, (Hamilton Company, Reno, Nevada, USA) 2 μl of cryopreserved trypanosome population diluted 1/5 in borate buffer were added to each well and incubated for 30 minutes at 37°C. The plates were examined for agglutination by dark illumination with x10 objective and x10 Periplan eyepiece.

2. 10. INDIRECT FLUORESCENT ANTIBODY TEST:

2. 10. 1. Formalin-fixed trypanosomes:

The method of Nantulya and Doyle (1977) was used in which 0.1 ml of freshly collected parasitaemic blood in microcentrifuge tubes was added to 0.9 ml of 1% formalin in PBS, and then incubated for 3 hours at 4°C with occasional agitation. The fixed trypanosomes were then centrifuged at 350 g for 10 minutes at 4°C. The supernatant discarded and pelleted trypanosomes were resuspended and washed twice in PBS by centrifugation at 350 g for 10 minutes at 4°C. Finally the trypanosomes were resuspended in 50 μl of heat inactivated foetal calf serum and thin smears were made on microscopic slides, air dried at room temperature for 10 to 15 minutes and reaction zones were made on the slides by a ball point paint marker (TEXPEN^{R}, Mark Tex Corp. USA). The

slides were wrapped in tissue papers, sealed in plastic bags and stored at 4°C in the presence of silica gel, as a desicating agent, until needed.

2. 10. 2. Acetone-fixed trypanosomes:

The method of Masterson *et al* (1988) was used in which thin smears of *T. evansi* infected blood on microscopic slides were air dried and fixed first by immersion in methanol for 6 minutes at - 20°C and then in acetone for 30 seconds at - 20°C. They were then washed by immersion in PBS for 5 minutes at room temperature, air dried and stored as above until needed.

2. 10. 3. Immunofluorescent Antibody Staining Procedure:

The stored slides were left to warm up at room temperature for 15 minutes before undiluted hybridoma culture supernatants or test sera at the appropriate dilution in PBS were added to the reaction zones. Positive and negative control serum and PBS were also included in each slide. 50 µl of each antibody solution were added to each reaction zone and the slides were incubated for 20 minutes at room temperature in a humid chamber. The unbound test reagents were washed off with a stream of PBS and the slides were immersed in PBS for 5 minutes and then in fresh PBS for a further 20 minutes. The slide edges were dried with tissue paper before 50 µl of fluorescein isothiocyanate (FITC)-labelled sheep anti-mouse IgG (The Binding Site Ltd, Birmingham, UK.) or FITC-labelled donkey anti-rabbit IgG (Scottish Antibody

Production Unit) was added to each reaction zone and allowed to react for another 20 minutes. The slides were washed as before and mounted in 50% glycerol in tris-saline, pH 9.0 for microscopy. The preparations were examined using a Diaplan fluorescence microscope with the x40 NPL fluorescence phase contrast oil objective and x10 Periplan GF eyepiece. The filter combinations used were a (2 x KP490 and 1 mm GG 455) excitation filter, a TK510 dichroic beam splitting mirror and a K515 suppression filter.

CHAPTER THREE

IDENTIFICATION AND CHARACTERISATION OF *TRYPANOSOMA EVANSI* (TREU 2165) COMPONENTS RELEASED DURING THE COURSE OF INFECTION AND PHYSICAL DISRUPTION

3. 1. INTRODUCTION:

Experiments in this Chapter were aimed at identifying suitable methods for the separation of the individual *Trypanosoma evansi* components and their antigenic characterisation as a preliminary step in identifying candidates for studies on the dynamics of these components during infection.

Most of the studies carried out on trypanosomal antigens (reviewed in Turner, 1982, Cross, 1990) have concentrated on the variable glycoprotein coat of the parasites probably due to its high degree of immunogenicity (Urquhart and Holmes, 1987), its role in protecting animals against reinfection (Wellde *et al*, 1981, Hall and Esser, 1984), its abundance (Cross, 1990) and the ease with which it can be purified from other parasite components (Cross, 1975, Strickler, *et al*, 1978, Reinwald *et al*, 1979, Clarke *et al*, 1984, Down *et al*, 1991).

To date little attention had been paid to the identification and characterisation of the non-surface antigens in the *Trypanozoon*, particularly in *T. evansi*. Uche (1989) studied antibody responses to both surface and non-surface antigens of *T. evansi* using a combination of SDS-PAGE and immunoblotting with serum from experimentally infected rabbits. He reported thirty soluble protein components as visualised by Coomassie Blue staining, of which 25 components were recognised as antigens by IgG antibodies in serum collected from infected rabbits. An association between trypanotolerance and invariant antigens was investigated by Shapiro and Murray (1982) who studied the immune response to *T. brucei* non-surface antigens in trypanotolerant N'dama and susceptible Zebu cattle during the course of infection using immuno-precipitation and SDS-PAGE. Eight antigens with molecular weights ranging from 300 k.Da to 20 k.Da were identified using these techniques. Animals that exhibited a capacity to control the disease responded to at least

one of three specific antigens of molecular weight of 110, 150, and 300 k.Da; the N›dama responded to more of these three antigens than did the Zebu. Animals that died of infection did not respond to any of the three specific antigens.

Results of previous studies indicate that the antigenic components of trypanosomes consist of a complex mixture of substances, with those identified so far being proteins or glycoprotein (Cross, 1975, Frommel and Balber, 1987, Muller *et al*, 1992, Authie *et al*, 1993). Most membranes including those of trypanosomes contain glycosylated molecules in which the carbohydrate is bound to protein (glycoprotein) or lipid (glycolipid). These glycoproteins and glycolipids often function as surface receptors or antigens. The trypanosomatidae contain a surface layer outside the cell membrane composed entirely of glycosylphosphatidylinositols (GPI) (Low, 1989, Cross, 1990b). These GPI molecules function as membrane anchors for a diverse group of proteins including the variant surface antigen of the trypanosomes (Ferguson *et al*, 1988). They probably comprise the major glycolipid class in the *Trypanosomatidae* and the molecules associated with them are involved in parasite infectivity and survival. In order to study the individual parasite components it is necessary to break the whole organism down into individual components. Numerous solubilisation methods have been used to break down complex biological structures into simpler components that might be studied more easily. Techniques include freeze-thawing, sonication, freeze-fracture, ionic and non-ionic detergent lysis with one of several detergents such as triton X-100, triton X-114, nonidet and sodium dodecyl sulphate.

All these solubilisation methods will result in the release or activation of the endogenous proteases present in the *Trypanosomatidae* which include lysosomal cysteine proteinases, cytosolic alkaline peptidases, acidic

proteinases, amino-peptidases and metalloproteinases (Lonsdale-Eccles and Grant, 1987; North *et al.*, 1983; Lonsdale-Eccles and Mpimbaza, 1986). The presence of these proteinases in lysates or extracts of trypanosomatidae can be controlled by including one or a number of inhibitors in the solubilisation medium. Solubilising intact trypanosomes can be effectively accomplished by boiling a suspension of trypanosomes in the presence of a reducing agent such as dithiothreitol or mercaptoethanol, and a 1% solution of SDS. This effectively solubilises both amphiphylic and hydrophylic molecules and boiling destroys protease activity. Proteins solubilised by this technique remain antigenic when tested by immunoblotting (Cardoso de Almeida and Turner, 1983, Frommel *et al*, 1988).

Experiments in this chapter were designed to compare two ways of extracting trypanosomal molecules, soluble materials produced by freeze-thawing trypanosomes and materials produced by boiling trypanosomes in the presence of SDS, which were evaluated by electrophoresis on polyacrylamide gels and also to examine the proteins in the trypanosomes after in situ trypsinisation to characterise their surface proteins as trypsin has been shown to remove the major surface VSG. The antigenicity of the parasite components was investigated against a range of serum from infected animals and animals immunised with *T.evansi* crude soluble materials.

3. 2. MATERIALS AND METHODS:-

3. 2. 1. Trypanosomes:

The history of the trypanosome TREU 2165 used in this study has been described in section 2.1.

3. 2. 2. Trypsinisation of *Trypanosoma evansi*:

Optimisation.

The effect of trypsinisation on *T. evansi* was investigated over a series of incubation periods of 15, 30, 45, 60, 75, 90, 105 and 120 minutes. To each of 8 samples containing $1x10^8$ trypanosomes in 1 ml PSG 20 µl of trypsin at a concentration of 5 mg/ml (Sigma Ltd., U.K.) was added and the tubes mixed by vortexing. Each tube was then incubated at 37°C for the appropriate time period. At the end of the incubation period a drop was removed from each tube and examined microscopically (x400 magnification) for trypanosome motility. To all tubes containing viable trypanosomes after trypsin treatment, 20 µl of trypsin inhibitor at a concentration of 5 mg/ml in distilled water (Sigma Ltd., U.K.) was added and the mixture incubated for a further 5 minutes at 37°C. The trypanosomes in each tube were then washed by centrifugation at 5000 g for 5 minutes in PSG. For each tube, the pellet of trypanosomes was resuspended in 0.5 ml PSG and inoculated each into a single mouse. Parasitaemia was monitored daily for up to 40 days using wet film preparations as described in section 2.5. The microhaematocrit centrifugation technique was also used to monitor the parasitaemia every three days.

Electron microscopy of trypsinised trypanosomes:

Intact trypanosomes and trypanosomes after being incubated with trypsin for 75 minutes and the trypsin neutralised with trypsin inhibitor, were fixed for 2.5 hours using 3% gluteraldehyde in 0.1M sodium cacodylate buffer pH 7.3 (Appendix 13). The trypanosomes were then washed in 0.1M sodium

cacodylate buffer by centrifugation at 2500 r.p.m. over 20 minutes with the buffer changed 3 times. They were then post-fixed in 1% osmium tetroxide in 0.1M cacodylate buffer for 45 minutes and washed in distilled water by centrifugation at 2500 r.p.m. over 20 minutes with the water changed 3 times. The trypanosomes were then dehydrated 1x10 minutes each in 50%, 70% and 90% acetone and 3 times 10 minutes in 100% acetone. They were then infiltrated with a 50:50 (v/v) araldite mix : Analar acetone (BDH, Leicestershire, U.K) for 30 minutes at room temperature, centrifuged at 2500 r.p.m. for 4 minutes and then infiltrated with the same mixture overnight at 60°C and centrifuged as before. Further infiltration was carried out 3 x 1 hour with araldite mix alone followed by two changes of araldite mix and accelerator (19:1, v/v) for one hour per infiltration. Finally trypanosomes were embedded in a 19:1 (v/v) araldite mix : accelerator for 48 hours at 60°C.

Sixty nm thick sections of trypanosomes were cut on a diamond knife with a Reichert OMU4 Ultramicrotome and mounted on 200 mesh copper grids. The sections were stained for 30 minutes in saturated uranyl acetate in 50% ethanol and then for 5 minutes in 2.76% lead citrate, pH 12.0 (Reynolds, 1963). Stained sections were viewed and photographed using a Philip 400 transmission electron microscope (x100000 magnification) at 100 KV.

3. 2. 3. *T. evansi* Soluble extract:

2.2×10^9 DEAE column separated trypanosomes of TREU 2165 were prepared as described in section 2.3. The separated trypanosomes were washed three times by centrifugation at 2650g for 20 minutes at 4°C with phosphate buffered saline glucose (PSG) pH 8.0 (Appendix 2). After the final wash the trypanosome pellet was resuspended in an equal volume of PSG and then subjected to three cycles of freezing to -80°C for 10 minutes followed by

thawing to room temperature for 10 minutes. The lysate was then centrifuged at 10000g for 45 minutes at 4°C using a Biofuge 17 RS centrifuge (Heraeus Sepatech, Essex, UK). The supernatant was removed and the protein concentration determined using the BCA^R technique according to manufacturer's instructions (Pierce Chemical Company, USA). The trypanosome soluble extract was aliquoted into 100 µl volumes and stored at –20°C until needed.

The trypanosome soluble extract prepared as described above was diluted 1:3 with SDS-sample buffer (Appendix 12) and heated to 100°C for 5 minutes. After cooling and centrifugation at 10000 g for 5 minutes, the sample was aliquoted into 200 µl volumes and stored at -20°C until required. Immediately before use the samples were heated again to 100°C for 3 minutes to redissolve any crystallised SDS and centrifuged as described above.

3. 2. 4. Whole trypanosome extract:

$2.2x10^9$ of washed trypanosomes collected from the DE52 column as above were diluted with an equal volume of SDS-sample buffer and heated to 100°C for 5 minutes. After cooling to room temperature and centrifugation at 10000 g for 5 minutes, the materials were aliquoted into 200 µl volumes and stored at -20°C until required. Immediately before use the samples were heated to 100°C for 3 minutes to redissolve any crystallised SDS.

3. 2. 5. Whole cell extract of trypsinised trypanosomes:

$2.2x10^9$ trypanosomes after being incubated with trypsin for 75 minutes and the trypsin neutralised with trypsin inhibitor were diluted with an equal volume of SDS-sample buffer and heated to 100°C for 5 minutes. After

cooling to room temperature and centrifugation at 10000 g for 5 minutes, they were aliquoted into 200 µl volumes and stored at -20°C until required.

3. 2. 6. Production of antibodies to *T. evansi*:

Antibodies to intact living trypanosomes (Infection serum):

Washed, column separated TREU 2165 trypanosomes were used to infect a rabbit by injection with $1x10^5$ trypanosomes in 1 ml PSG through the marginal ear vein. Prior to infection 2 ml of blood were collected from the rabbit for production of pre-infection serum. Parasitaemia was monitored daily using wet film preparations from the marginal ear vein until the experiment was finished 21 days post-infection.

Ten ml of blood was collected from the marginal ear vein of the rabbit on days 2, 5, 9, 14 and 21 after infection. Serum was obtained from the blood as described in section 2.6. The experiment was terminated 21 days after infection, by treating the rabbit with a single i.m. injection of diaminazine aceturate (Berenil^R, Hoechst Lab., Germany) at dose rate of 7 mg / Kg body weight.

Antibodies to trypsin-treated trypanosomes

$1x10^7$ trypanosomes treated with trypsin (20µl @ 5mg/ml) for 75 minutes were suspended in 1 ml PSG and inoculated intravenously (i.v.) into one rabbit. The inoculation was repeated on days 28 and 57 with $1x10^7$ freshly prepared trypsinised trypanosomes. On each occasion two mice were also inoculated intraperitoneally with $1x10^7$ trypsinised trypanosomes in 0.5 ml PSG to serve as infection controls. The mice were monitored for parasitaemia daily using wet blood films of tail blood. The microhaematocrit centrifugation technique was used to monitor parasitaemia in the rabbit at time of collection

of blood for serum and in mice at the end of the experiment on day 63 after injection.

Blood was collected from the rabbit on days -1, 7, 9, 21, 35, 40, 50 and 63 after injection. Serum was prepared as described in section 2.6.

Antibodies to *T. evansi* soluble extract:

A rabbit was immunised with *T. evansi* soluble extract, prepared as described in section 3.2.3, using an immunisation regime adapted from Harlow and Lane (1988). The rabbit was first injected subcutaneously at eight different sites along its back with a total of 200 μg (800 μl) of the freeze-thawed *T. evansi* soluble extract emulsified in Freund's Complete adjuvant (FCA). The emulsion was prepared at a ratio of antigen to adjuvant of 1:2 using 5 ml glass syringes connected through a luer fitting. The antigen was first injected into the adjuvant and the mixture was then passed repeatedly between the syringes until the emulsion became thick and creamy. The rabbit was then boosted by s/c injection with a further 100 μg (400 μl) of the same materials in incomplete Freund's adjuvant (FIA) at 28 and 56 days post-immunisation. A final boost of 100 μg soluble extract in PSG was administered intravenously to the rabbit 68 days after the first immunisation.

The rabbit was bled for production of serum on days -1, 7, 10, 21, 34, 39, 49, 61, 67 and 75. The serum was prepared as described in section 2.6.

3. 2. 7. SDS-PAGE electrophoresis of *T. evansi* extracts:

Fifty microlitre of *T. evansi* freeze-thawed soluble extract containing 45μg protein, 50μl SDS-solublised whole trypanosome extract equivalent to $1.1x10^8$ trypanosomes and 50μl SDS-solublised trypsinised trypanosomes extract equivalent to $1.1x10^8$ trypanosomes were subjected separately to SDS-PAGE gradient gel electrophoresis as described in section 2.7. Visualisation of

the gel protein banding pattern was by Coomassie Blue stain as described in section 2.7.

3. 2. 8. Detection of antigenic components of *T. evansi*

T. evansi soluble extract (50 µl, 45 µg), whole trypanosome extract (50 µl, $1.1x10^8$ trypanosomes) and trypsinised trypanosomes extract (50 µl, $1.1x10^8$ trypanosomes) were subjected to SDS-PAGE. Proteins from the gel were then transferred to a nitrocellulose membrane as described in section 2.7.1. The reactivity of the separated proteins was examined by incubation of the membrane with antisera from the two rabbits inoculated with intact or trypsinised trypanosomes and from the rabbit immunised with soluble extract of the intact trypanosomes. The serum samples collected from each rabbit were pooled equally and then diluted to 1/50 in blocking buffer, pH 7.4 (Appendix 7) before used in the assay. The assay was performed as described in section 2.7.1.

3. 2. 9. Enzyme-linked immunosorbent assay (antibody-ELISA) for trypanosome antibodies:

Serum samples from the two rabbits inoculated with intact or trypsinised trypanosomes and from the rabbit immunised with soluble extract of the intact trypanosomes were assayed for *T. evansi* antibodies by ELISA. Each serum sample was tested at a dilution of 1/50 against soluble extracts prepared by freeze-thawing from trypsin-treated and untreated *T. evansi* trypanosomes at a coating dilution of 1/80. Pre-immunisation serum from each rabbit at a dilution of 1/50 was included in the assay as negative controls. All

serum samples were tested in duplicate and the antibody-ELISA was performed as described in section 2.8.

3. 3. RESULTS:

3. 3. 1. Trypsinisation of *T. evansi* :

Trypanosomes remained intact and motile after incubation with trypsin for periods of up to two hours. The incubation of the trypanosomes with trypsin for up to 45 minutes did not affect their infectivity as parasites were found in mouse blood three days following i.p. inoculation (Table 3. 1). Incubation for one hour, however, reduced the infectivity of the parasite as pre-patent period was extended to ten days (Table 3. 1). While incubation of trypanosomes with trypsin for periods of 75, 90, 105 and 120 minutes completely abolished their infectivity to mice as trypanosomes were not detected by 40 days after inoculation.

Electron microscope sections prepared from untreated trypanosomes were seen to possess a thick (20 nm), compact and dense surface coat overlying the plasma membrane. In the sections prepared from trypanosomes incubated with trypsin for 75 minutes, the surface coat was removed, leaving behind the plasma membrane which was sometimes difficult to resolve but the microtubules appeared normal. Representative photograph of these sections shown in Figure 3. 1a and Figure 3. 1b.

Table 3. 1

Parasitaemia in mice inoculated with trypanosomes incubated with trypsin for periods ranging from 15 to 120 minutes

Number of trypanosomes per 20 microscopic fields at x400 magnification.

Days	15 minutes incubation	30 minutes incubation	45 minutes incubation	60 minutes incubation	75-120min incubation
1	0	0	0	0	0
2	0	0	0	0	0
3	1	1	1	0	0
4	N	N	N	N	0
5	N	N	N	N	0
6	4	9	3	0	0
7	59	100	6	0	0
8	120	64	1	0	0
9	200	0	0	0	0
10	D	5	2	1	0
11-40	-	N	N	N	0

N = Not done.

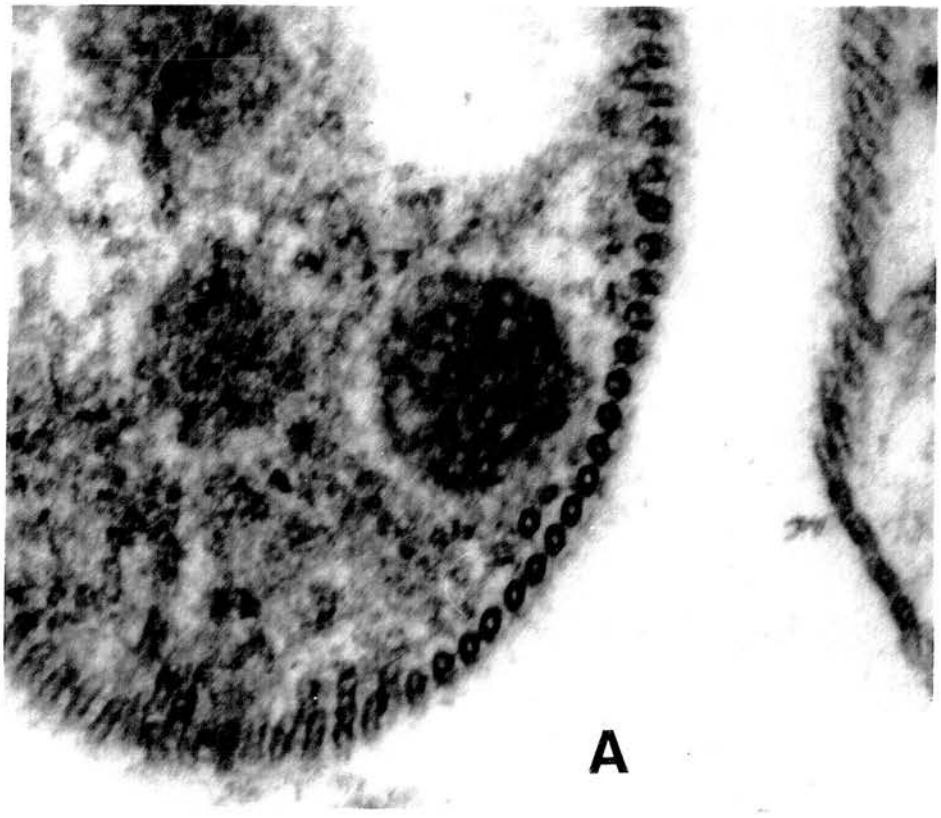
D = Died.

FIGURE 3. 1

Transmission electron micrograph of section through surface of body of *T. evansi*(TREU 2165). 1 mm ≡ 10 nm.

a- 75 minutes trypsin-treated trypanosomes lacking a surface coat.

b- Intact non-treated trypanosomes possessing a surface coat.



A

B

3. 3. 2. Parasitaemia In Inoculated Rabbits:

The parasites were first detected on day 5 following injection of rabbit with the living trypanosomes. Parasitaemia fluctuated, with parasites detected only on days, 12, 13, 14, 15, 16 and 21 post-infection.

Trypanosomes were not detected by the microhaematocrit technique during the 63 days of immunisation regime in the rabbit inoculated with the trypsin-treated trypanosomes. Control mice inoculated with these trypanosomes also did not show parasites in their circulation.

3. 3. 3. SDS-PAGE separation of *T. evansi* materials:

Slight difference was seen in the number of the protein bands resolved by Coomassie blue stain of the whole trypanosome extract and the soluble extract. The highest number of individual proteins were seen in the whole trypanosome extract with a total of 39 individual protein bands with molecular weight ranging from 158.6 k.Da to about 9.1 k.Da (Figure 3.2, lane No. 2). Two of these protein bands with molecular weight of 146 k.Da and 142 k.Da were absent from the soluble extract which contained 37 proteins (Figure 3.2, lane No. 3). Otherwise both extracts had similar banding patterns with high molecular weight components in the range of 158.6 k.Da - 125.1 k.Da, showed the lowest staining intensities.

Of the 39 bands in the whole extract only 32 polypeptide bands were visible by Coomassie blue staining of the trypsinised trypanosomes extract in the size range of 96 k.Da to 9.1 k.Da (Figure 3.2, lane No. 4). Seven of the protein bands present in the whole trypanosomal extract, were absent from the trypsinised trypanosomes extract. Five of these bands were in the high molecular weight part of the gel (158.6 - 125 k.Da) with the other two at 58.6

and 52 k.Da. Furthermore only few proteins dominated by a 42 k.Da component showed a similar staining intensity to that in the other two extracts. The staining intensity of the other proteins in the trypsinised trypanosomes extract appeared to be lower than that of the other two extracts despite equivalent amounts of trypanosomes.

3. 3. 4. Immunological characterisation of *T. evansi* proteins (Table 3. 2):

Reactivity of infection serum:

Pooled serum collected from the infected rabbit for up to 21 days after infection recognised a total of seventeen antigenic components ranging in molecular size between 172 k.Da to 21.2 k.Da (Figure 3. 3. lane No. 1 and table 3. 2.) in the three trypanosomal preparations used in this study. Of the 39 protein components present in the whole trypanosome extract 16 reacted with the infection serum. Twelve of these antigens were also recognised by this serum in the soluble extract. Five antigens of 65.5, 58.6, 31.5, 30 and 21.2 k.Da present in the whole extract were absent from the soluble extract with one 44 k.Da component present only in the soluble extract. The serum recognised 7 components in the trypsinised trypanosome extract with corresponding antigens in both whole and soluble extracts while a 65.5 k.Da component was common to the trypsinised and whole trypanosomes extract.

Reaction of hyperimmune serum:

Serum raised to *T. evansi* soluble extract recognised a total of twenty protein components in the three trypanosomal preparations used in this study with a molecular weight ranging from 166.1 k.Da to 26.6 k.Da (Figure 3. 3. lane No. 2 and table 3. 2). Ten antigens recognised during 21 days of infection were also recognised by the hyperimmune serum. The majority of the antigens recognised by hyperimune serum were present in the soluble extract with 16

components recognised by the serum. Seven of these 16 antigens were also present in the whole cell extract and one 58.6 k.Da component was present only in the whole extract. The serum recognised 4 of the above 16 components in the trypsinised trypanosome extract which also contained two additional components of 54.7 and 26.6 k.Da.

Reactivity of trypsinised trypanosomes antiserum:

Serum raised to the trypsinised trypanosomes did not recognise any protein component on any of the three trypanosomal preparation (Figure 3. 3 lane No. 3).

FIGURE 3. 2

Total protein profile of *T. evansi*(TREU 2165) fractionated on SDS-PAGE and stained with Coomassie Blue.

1. Molecular weight markers.
2. Whole cells extract.
3. Soluble extract.
4. Trypsinised trypanosomes extract.

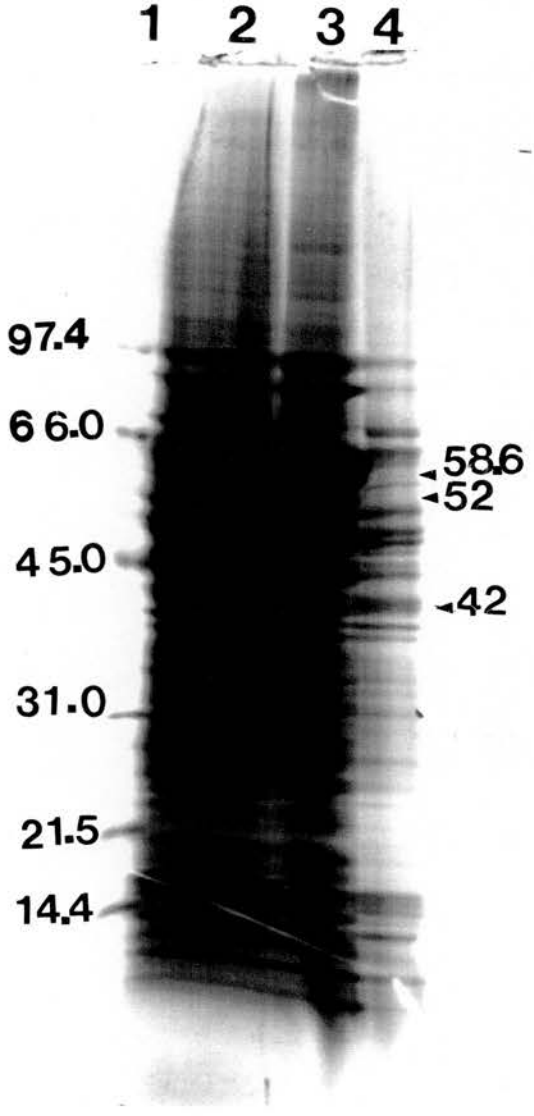

1
2
3
4
97.4
66.0
45.0
31.0
21.5
14.4
58.6
52
42

FIGURE 3. 3

T. evansi(TREU 2165) proteins identified by polyclonal polyspecific antisera raised in rabbits against intact living trypanosomes, trypsin-treated trypanosomes and soluble extract and probed by western immunoblotting.

MM. Molecular weight marker.

1. Serum raised against intact living trypanosomes.

2. Serum raised against soluble extract.

3. Serum raised against trypsin-treated trypanosomes.

a- Soluble extract.

b- Trypsin-treated trypanosomes extract.

c- Whole cell extract.

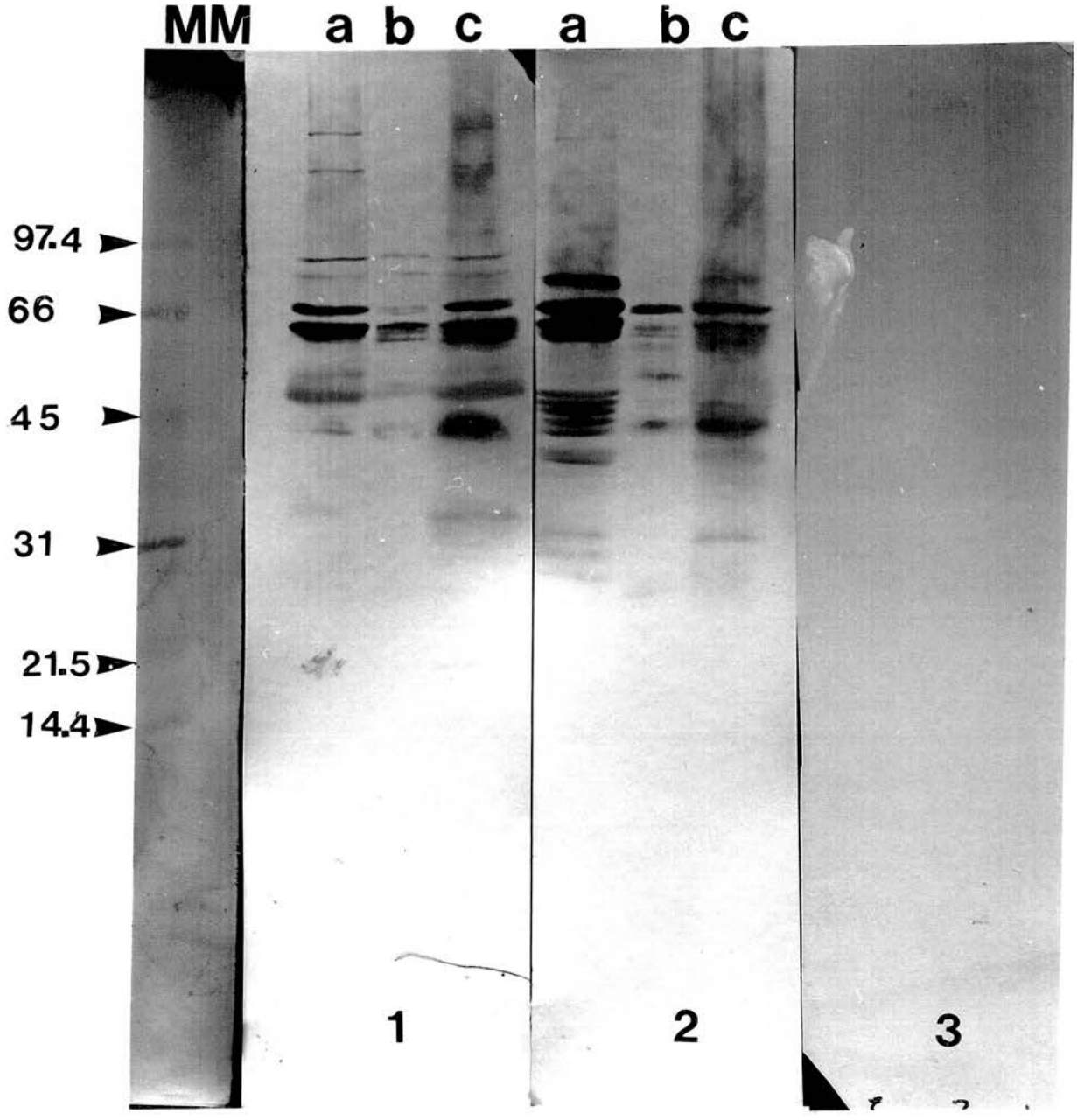

MM
a
b
c
a
b
c
97.4
66
45
31
21.5
14.4
1
2
3

Table 3. 2

T. evansi antigens recognised by infection serum and hyperimmune serum to *T. evansi* soluble extract.

		Antigenic during infection when tested against			Antigenic as soluble extract when tested against		
	M.wt in k.Da	Whole parasite extract	Soluble extract	Trypsinised whole parasite extract	Whole parasite extract	Soluble extract	Trypsinised whole parasite extract.
1	172	Yes	Yes	No	No	No	No
2	139	Yes	Yes	No	No	Yes	No
3	90	Yes	Yes	Yes	No	No	No
4	83.7	Yes	Yes	Yes	No	No	No
5	70.1	Yes	Yes	Yes	Yes	Yes	Yes
6	65.5	Yes	No	Yes	No	No	No
7	63.3	Yes	Yes	Yes	Yes	No	Yes
8	60.0	Yes	Yes	Yes	No	Yes	Yes
9	58.6	Yes	No	No	Yes	No	No
10	52	Yes	Yes	No	No	Yes	No
11	46.4	Yes	Yes	Yes	No	Yes	No
12	44	No	Yes	No	No	No	No
13	42	Yes	Yes	Yes	Yes	Yes	Yes
14	33.2	Yes	Yes	No	No	No	No
15	31.5	Yes	No	No	Yes	Yes	No
16	30.0	Yes	No	No	No	Yes	No
17	21.2	Yes	No	No	No	No	No
Total bands		16	12	8	5	8	4

Table 3. 2 continued

T. Evansi antigens recognised by infection serum and hyperimmune serum to *T. evansi* soluble extract.

		Antigenic during infection when tested against			Antigenic as soluble extract when tested against		
	M.wt k.Da	whole cell extract	soluble extract	trypsin wc extract	whole cell extract	soluble extract	trypsin wc extract.
1	166.1	No	No	No	No	Yes	No
2	104	No	No	No	No	Yes	No
3	80.8	No	No	No	Yes	Yes	No
4	54.7	No	No	No	No	No	Yes
5	47.8	No	No	No	Yes	Yes	No
6	41.8	No	No	No	No	Yes	No
7	38.9	No	No	No	Yes	Yes	No
8	38.1	No	No	No	No	Yes	No
9	28.1	No	No	No	No	Yes	No
10	26.6	No	No	No	No	No	Yes
Total bands		0	0	0	3	8	2

3. 3. 5. Antibody response measured by ELISA:

The rabbit immunised with trypsinised trypanosomes did not show a measurable antibody response by ELISA. Absorbance values of the serum collected from this rabbit post-inoculation did not exceed that of the pre-inoculation serum when tested against the soluble extracts of the trypsin-treated and untreated trypanosomes (Figure 3. 4). Sera from the infected rabbit and rabbit immunised with the soluble extract of the intact parasite, however, reacted with the trypsin-treated trypanosomes when they were used as antigen in a similar way to their reaction with the untreated trypanosome extract. Absorbance values with trypsinised trypanosomes as antigen were, however, consistency lower than those with antigen from intact trypanosomes (Figure 3.4).

3.4 DISCUSSION

The whole trypanosomes extract contained the largest number of the parasite proteins compared to the other two trypanosomal preparations used in this study. A total of 39 proteins were revealed by the Coomassie stain in this extract. Two of these proteins were not released by aqueous solubilisation as they were absent from the soluble extract and contained 37 proteins. The resistance of these proteins to aqueous solubilisation and their release from the parasite by SDS detergent lysis indicates a possible membrane association as detergent lysis is known to solubilise the membrane proteins by replacement of the lipid bilayer with a micelle of detergent (Goding, 1986). The trypsinised trypanosomes extract contained the lowest number of the parasite proteins, with seven bands absent from the trypsinised extract compared to that in the whole trypanosomes extract. Although trypsin digestion is reputed to cleave mainly the VSG from the parasite surface (Cross, 1975), other proteins were reported to be cleaved during trypsinisation (Frommel et al, 1988). Possibly, in the present study, these molecules possessed trypsin sensitive sites, arginine and lysine (Stryer, 1988), and were released from the parasite together with the surface coat during the process of trypsinisation. Of the proteins absent after trypsinisation, two of 58.6 and 52 k.Da fall within the 53 to 63 k.Da range reported for VSG molecules of *T. evansi* (Richards, 1984) and the 53 to 65 k.Da range for *T. brucei* (Cross, 1975, Johnson and Cross, 1977, Hoeijmakers *et al*, 1980).

Twenty seven components of *T. evansi* were found to be antigenic in the present study depending on the extraction procedure. Seventeen of these components were recognised as antigens during infection as revealed by their reaction with the infection serum collected for up to 21 days after infection. The number of the parasite antigens recognised during the infection in the

present study was less than that reported by Uche (1989) who reported up to 25 antigenic components after 28 days infection with *T. evansi* in rabbits. This difference could be due to the difference in the duration of the infection as after 28 days the animal could be expected to be exposed to wider range of antigens than at 21 days.

The molecular weights of the *T. evansi* antigens identified in the present study ranged from 172 k.Da to 21.2 k.Da although the upper molecular weight limit of the parasite proteins revealed by Coomassie Blue Stain was 158.6 k.Da. This difference is possibly due to the fact that Western immunoblotting is a more sensitive detection system than Coomassie Blue. Coomassie Blue has a detection limit of 1 μg protein (Harlow and Lane, 1988), while as little as 100 pg of protein can be detected by the immunoblotting (Towbin *et al*, 1979).

Most of the antigens recognised during the course of infection were also immunogens when presented as a soluble extract as they reacted with both the infection serum and the serum raised against the parasite soluble extract Differences were, however, detected in the antigenicity of the parasite components released during infection and by physical disruption. Some of the parasite components which were not antigenic during the course of infection acted as antigens when presented to the host in soluble extract. This difference in the antigenicity of these parasite materials could be accounted for by difference in the way they were released from the parasite (Barriga, 1981) and the way in which they were presentated to the host eg immunogenicity of those incorporated with adjuvant will be increased due to their presentation in an aggregated form (Goding, 1986).

Twelve of the antigenic components of *T. evansi* recognised by the host during the 21 days of infection in the present study were soluble proteins as

recognised by the infection serum in the soluble extract, while only 5 were insoluble proteins possibly of membrane origin as they were present only in the whole trypanosome extract prepared by the detergent lysis. Nine of the 17 antigens recognised by the infection serum were sensitive to trypsin as they were absent from the trypsinised trypanosomes extract. This finding suggests that these antigens possess trypsin sensitive sites and are present in the surface of the parasite as the trypanosomes were viable and motile after the trypsinisation. Surface antigens other than the VSG have been reported in the trypanosomes (reviewed in Overath *et al*, 1994).

Trypanosome infectivity was completely abolished when mice were inoculated with motile trypsin-treated trypanosomes after incubation with trypsin for periods greater than one hour The loss of the trypanosomes infectivity following trypsin digestion reported in the present study is in agreement with that reported by Cross (1975). This had been attributed to the activation of complement by the alternative pathway caused by the exposure of the plasma membrane of the parasite following the removal of the surface coat (Ferrante and Allison, 1983). When the trypanosomes are covered with C3b they are phagocytosed by macrophages (Newton, 1981), suggesting the surface coat had been removed. Electron microscopy of the trypanosomes after 75 minutes incubation (Figure, 3. 1a) in the present study showed a uniform removal of the surface coat. This period of incubation was chosen as an optimum incubation period for the release of the surface coat of TREU 2165, since shorter periods did not completely remove the parasite coat as indicated by infectivity to mice, longer periods of incubation, on the other hand, might lead to lysis of the parasite (Cross and Johnson, 1976).

Trypsin-treated trypanosomes did not stimulate antibody responses when injected into a rabbit. Serum raised against these trypsinised

trypanosomes in the present study did not recognise any antigenic components of the parasites in the immunoblots. This was supported by the results from the antibody-ELISA where serum samples collected from the rabbit immunised with the trypsinised trypanosomes did not react with the freezed-thawed trypsin-treated or untreated trypanosomes when used as antigen.. However serum collected on day 63 post-inoculation with trypsinised trypanosomes showed a slight rise in absorbance level of the antigen-antibody reaction but was still within the negative control level. The reactivity of the freeze-thaw, trypsin-treated trypanosomes with serum raised against the intact living trypanosomes and the soluble extract was lower compared to that of the untreated trypanosomes. This could be accounted for by difference in the antigen composition in the two trypanosomal preparations with the trypsinised trypanosomes lacking the surface coat antigen which is the the most abundant trypanosome antigen (Cross, 1990).

The lack of antibody response to trypsin-treated trypanosomes suggests that, the surface coat antigen might be important in the initiation of the humoral immune response against the non-surface antigens This might take place through direct activation of B lymphocytes without the involvment of antigen-presenting cells such as macrophages. The role of macrophages in the presentation of the trypanosomes antigens has been reported as negligible (Paulnock *et al*, 1988). Alternatively, the trypsinised trypanosomes,unlike the intact trypanosomes, did not replicate within the host body as indicated by their absence in the circulation of the rabbit and mice inoculated with these trypsinised trypanosomes. This would limit the amount of antigens released from these trypsinised trypanosomes compared to the continuous stimulation of the immune system in animals infected with the intact trypanosomes. (De Raadt, 1974, Shapiro and Murray, 1982, Uche, 1989).

The present study has identified a number of *T. evansi* components which acted as antigens during the first 21 days of infection, the majority of which were soluble proteins. Some of these antigens were trypsin-sensitive in the intact organism suggesting a surface assoctiation. Two soluble antigens were selected for further study - a trypsin sensitive 52 k.Da and a 42 k.Da antigen which was recognised as a strong antigen in the three *T. evansi* preparations investigated in this study. Both antigens were also good immunogens when administered as soluble extracts making them suitable for use in a range of purification methods.

CHAPTER FOUR

PRODUCTION OF POLYCLONAL ANTIBODIES TO A 42 AND 52 K.DA *T. EVANSI* ANTIGENS

THE AIM

The aim of this section was to raise specific antibodies to two soluble *T. evansi* antigens, the 52 k.Da and the 42 k.Da antigens identified in section three as major components in the immune response to *T. evansi* infection. The antibodies will then be used in assays for the detection of the corresponding antigen.

4. 1. INTRODUCTION:

Individual antigens purified to homogeneity is the principal requirement for the production of the highly specific antisera needed for use in immunoassays as the sensitivity of these assays is dependent on the specific activity and high avidity of the antibody.

Prior to purification of individual parasite components, the organism has to be obtained in a pure form separated from host materials. Unlike many haemoflagelates, trypanosomes can be easily separated from host blood by ion-exchange chromatography using DEAE cellulose (Lanham and Godfrey, 1970). The trypanosomes themselves like all protozoa consist of a mixture of chemical components. The initial separation of individual antigens from this complex mixture is usually based on differences in physicochemical properties of the individual antigens. Trypanosomes antigens are predominantly proteins (Shapiro and Murray, 1982, Muller *et al*, 1992, Authie *et al*, 1993) or glycoproteins and classified as either surface (reviewed by Turner, 1982, Cross, 1990 and Overath *et al*, 1994) or non-surface (Strickler *et al*, 1978, Frommel and Balber, 1987). The majority of studies carried out on the purification of trypanosomal antigens has concentrated on the major surface glycoprotein - the variant specific glycoprotein. This is probably due to VSG's

high degree of immunogenicity, its role in protecting animals against reinfection (Wellde *et al*, 1981, Hall and Esser, 1984), its abundance as it represents 10% of total parasite protein (Cross, 1990) and the relative ease with which it can be purified from other parasite components. Most of the purification methods involve some form of chromatographic separation including ion-exchange chromatography (Clarke *et al*, 1984), size exclusion chromatography (Down *et al*, 1991) and, to a lesser extent, affinity chromatography using lectins (Johnson and Cross, 1977, Strickler *et al*, 1978, Reinwald *et al*, 1979, Turner, 1982) or antibodies (Pearson and Anderson, 1980).

Although many of the above purification methods can be applied equally to the non-variant protein antigens, their number and heterogeneity necessitates the development of different methodologies for each antigen and possibly in combination. One relatively simple method for purification of antigens is based on their separation by SDS-polyacrylamide gel electrophoresis (Harlow and Lane, 1988) in which proteins are separated according to their molecular size and subsequently eluted from the gel. This method however, is only usable if the polypeptides of interest can be resolved as unique bands on such gels. This method has the overall advantage of speed and avoids the breakdown of antigens by exposure to different pH and salt concentration as in chromatographic methods. Sample preparation for SDS-PAGE, however, involves the use of a strong ionic detergent and reagents such as DTT that can lead to denaturation and changes in epitopes. Nevertheless, SDS-denatured antigens often retain their antigenicity and induce good antibody responses (Stumph *et al*, 1974) and this method has been used to analyse the immune response to a wide range of organisms (Strauss *et al*, 1975, Carrol *et al*, 1978, Boulard and Lecroisey, 1982).

A crucial part of using SDS-PAGE for antigen isolation is the efficient localisation of the target antigen in the gel while avoiding any further denaturation that can be caused by conventional staining. Localisation methods include, the use of a separate reference lane which is stained and used to localise the band of interest in rest of the gel. This method however, is only suitable for purification of proteins that are well separated from each other under condition of SDS-PAGE (Harlow and Lane, 1988) as small errors in aligning the reference lane and the rest of the gel can lead to contamination of the excised gel band. Alternative localisation methods involve whole gel staining with Coomassie blue, sodium acetate or copper chloride to locate the bands of interest. This method ensures the unequivocal identification of the target component with a minimum risk of contamination from nearby bands, but runs the risk of altering epitopes due to combination with stain.

For raising antibodies to the target antigen the excised gel containing the antigen can be fragmented by repeated passage through a syringe and then injected into animals for production of antibodies. Fragmentation of high acrylamide concentration gels is difficult and the technique does not allow for an accurate quantification of the protein injected into the animal during immunisation or incorporation with adjuvants Another disadvantage of this method is that the release of antigens from the acrylamide is sometimes too slow to allow sufficient stimulation of the immune system (Goding 1986). More effectively proteins trapped in the gel can be made to migrate out of the gel under the influence of an electric field and the electrophoretically eluted protein can then be used for immunisation (Harlow and Lane, 1988). Up to 90% of the amount of protein contained in the excised gel can be obtained by this method (Goding, 1986). A major advantage of this method is that, the

concentration of the eluted protein can be measured and the antigen can be incorporated with an adjuvant before injection into animals.

The immunisation regime and the animal species influence greatly the type of response and antibody characteristics. Sustained high levels of high affinity antibodies are usually produced by repeated injection of animals with the same antigen. Soluble protein antigens in their native state are often poorly immunogenic (Dresser, 1962) and require incorporation with an adjuvant at an early stage of immunisation which presents them in an aggregated form, thus, enhancing their immunogenicity. The most popular adjuvants include aluminium salts or mineral oils (Freund's adjuvant) both of which act by lowering the rate of elimination of the immunogen by forming depots. This results in a prolonged persistence of the immunogen in tissues and thus a continuous stimulation of the immune system (Tijssen, 1985). The intervals between booster injections should be timed to ensure that rapid clearance of the antigen by the circulating antibodies does not occur. Hyperimmune sera produced this way usually contain high levels of IgG of specific antibody with high affinity to the immunogen due to maturation of the immune response (Harlow and Lane, 1988). This makes them particularly suitable for use in immunoassays which require high affinity antibodies.

In the present study a number of methods were investigated for the purification of two *T. evansi* antigens including SDS-PAGE and several chromatographic methods.

4. 2. MATERIALS AND METHODS:

4. 2. 1. Trypanosomes:

Three populations of *T. evansi* (TREU 2165, 2222 and 2257) representing two stocks of the parasite, one stock of *T. brucei* (TREU 2185) and one stock of *T. vivax* (TREU 2130) were used in this part of the study. The history of *T. evansi* stocks is presented in section 2.1. *T. brucei* was acquired by C.T.V.M. from the East African Trypanosomiasis Research Organisation (E.A.T.R.O.) in 1964 and *T. vivax* as the rodent adapted Wellcome strain of *T. vivax* acquired by C.T.V.M. in 1964.

4. 2. 2. preparative gel electrophoresis:

A total of ten SDS-PAGE gels (160 mm x 180 mm x 1.5 mm) were used to provide the materials for immunisation. A *T. evansi* soluble extract was prepared for SDS-PAGE as described in section 3.2.2. and separated on 7-20% gradient SDS-PAGE gels. 100 µl samples of the soluble extract were loaded in each of the 14 wells cast into the gel and electrophoresis was performed as described in section 2.7. After electrophoresis and Coomassie blue staining of the whole gel the 42 and 52 k.Da *T. evansi* proteins were located in the gel by reference to a molecular weight standard lane (Molecular weight markers, Pharmacia) electrophoresed in the same gel. Portions of acrylamide containing the 42 and 52 k.Da proteins were cut with a scalpel from each gel and placed in the glass tubes of an electro-elution device (Model 422 Electro-Eluter, Bio Rad, USA) set up according to the manufacturer›s instructions as described in section 2.7.2. The proteins were then electrophoretically eluted from each excised gel portion at a constant current

of 10 mA per glass tube for 5 hours. A total of 600 µl containing the appropriate protein was collected from the collection capsule of each tube. The homogeneity and purity of proteins eluted from each portion was assessed by SDS-PAGE using 10% homogeneous acrylamide mini-gels (8 x 10 cm, 0.75 mm thick; Mini-Protein^{R} II, Bio-Rad, USA) prepared according to manufacturer›s instructions. Proteins were detected in these gels with a silver stain kit (Silver Stain Plus kit, Bio-Rad, USA) following the manufacturer's instructions.

For each antigen, all electro-eluates were pooled, transferred to dialysis tubes (molecular weight cut-off 12,000 daltons; Sigma chemical Co. St. Louis, USA) and dialysed overnight against PBS. The total protein concentration of the dialysed proteins was measured using a BCA^{R} test kit (Pierce, USA). The 42 and 52 k.Da proteins were then concentrated approximately 6-fold using a Centrifugal Ultrafiltration System (Sartorius Ltd., Germany) in which 2.5 ml of eluate were placed in an outer centrifuge tube (92 x 14 mm. Centrisart L, Sartorius Ltd.). An inner tube (floater) with a cellulose triacetate ultrafilter (molecular weight cut-off of 5000 daltons) sealed to its base was then slipped into the outer polystyrene tube. The sample was then centrifuged at 2500 g for 30 minutes at 4^{o}C. After removing the inner tube containing the ultrafiltrate, the concentrated protein in 380 µl PBS was collected and stored at - 20^{o}C until needed.

4. 2. 3. Separation of antigens by High performance liquid chromatography

$3x10^{8}$ column separated and washed *T. evansi* (TREU 2165) trypanosomes were suspended in 4 ml of ice-cold 0.015M phosphate buffer, pH 8.0 (Appendix 14) containing N-CBZ-L-Phenylalanine Chloromethyl Ketone (ZPCK, 0.4 mg/ml). Four milligrams of n-Octyl β-D- Glucopyranoside (OGP) was then added directly to the trypanosomes suspension, mixed gently

for 2 minutes and then centrifuged at 20,000 g for 35 minutes at 10°C in a Beckman ultracentrifuge (L8-M, Beckman, USA) using a fixed head 65K rotor. The supernatant was carefully removed and subjected to fractionation by ion-exchange high performance liquid chromatography (HPLC).

Ion-exchange HPLC of the detergent solublised fraction was carried out using a 75 x 7.5 mm Spherogel TSK DEAE-5 PW column (Beckman, USA). One ml of the solublised trypanosome materials was injected onto the column in 20 mM tris buffer, pH 7.0 (buffer A, Appendix 15) using a 1 ml sample loop. Proteins were eluted from the column using a continuous gradient generated using two buffers (a 20 mM tris, pH 7.0 start buffer and a high salt buffer containing 20 mM tris buffer pH 7.0 with 240 mM NaCl, Appendix 16) at a flow rate of 1 ml/minute over a period of 30 minutes. The absorbance of the column eluate was monitored continuously at 280 nm and the eluate collected into 1.7 ml microcentrifuge tubes at one minute intervals.

Five fractions representing the major ion-exchange elution peak collected during the first 3-7 minutes were analysed using a 8-25% gradient Phast gel (Pharmacia, USA) SDS-PAGE system. Samples from each fraction were diluted with twice their volume in SDS-sample buffer (Appendix 12), heated to 100°C and centrifuged at 10000g for 5 minutes. Four µl of each sample were then applied to the gel and electrophoresis carried out at 250 V for a total of 80 volts hour at 15°C, for 45 minutes. After electrophoresis the gel was removed from the apparatus and the separated proteins were visualised by silver staining in the development chamber of the Phast unit. This staining process consisted of the gel being incubated with a solution of 50% ethanol and 10% acetic acid for 2 minutes at 50°C, proteins were then fixed with 8.3% gluteraldehyde for 6 minutes at 50°C and washed twice in 18 Mohm water each for 2 minutes. The proteins were then incubated with 0.25% silver nitrate

for 13 minutes at 40°C and washed twice in the 18 Mohm water for 0.5 minutes each at 30°C. The reacted proteins were then incubated with developer solution for periods of 0.5 minute and 4 minutes respectively at 30°C; developer solution contained 12.5 g of sodium carbonate and 0.2 ml formaldehyde in 500 ml water. Silver stained gels were then incubated for 2 minutes at 50°C with 5% acetic acid, and finally for 3 minutes with a mixture of 10% acetic acid and 10% glycerol. Stained gels were air dried for one hour before photography.

4. 2. 4. Size exclusion chromatography of the ion-exchange fractions:

The 5 fractions collected from the HPLC ion-exchange column were pooled together and re-fractionated according to molecular size by size exclusion HPLC (Welling and Welling-Wester, 1989). One ml of the pooled sample was applied in 0.1 M Na_2SO_4, 0.1 M KH_2PO_4, 0.001 M NaN_3, pH 7.0 buffer (Appendix 17) to a 300 x 7.5 mm Biosep-SEC-S 300 column (Phenomenex, UK) using a 1 ml sample loop. Samples were eluted from the column over a period of 30 minutes using a flow rate of 0.65 ml/min. The absorbance of the column eluate was monitored continuously at 280 nm and the eluate fractions collected into 1.7 ml microcentrifuge tubes at one minute intervals. Nine fractions were collected between 12 and 20 minutes of the chromatography run representing the major elution peak. These fractions were analysed by SDS-PAGE using a homogenous 15% acrylamide mini-gel (8 x 10 cm, 0.75 mm thick, Mini-Protein^R II, Bio-Rad, USA) prepared and run according to manufacturer's instructions. Proteins were detected in the gel with a silver stain kit (Silver Stain Plus kit, Bio-Rad, USA) following the manufacturer›s instructions.

4. 2. 5. Lectin-affinity chromatography:

The pool of 5 fractions eluted from the HPLC ion-exchange column was subjected to lectin affinity chromatography using Concanavalin A sepharose 4B (Con A sepharose, Sigma Ltd. UK). Prior to chromatography 4 ml of the Con A-sepharose gel was equilibrated with 10 ml Con A binding buffer (Appendix 18). The sample (3 ml) was mixed with 7 ml of binding buffer and then allowed to pass through a column prepared from 4 ml of Con A-sepharose. The column eluate was passed back through the column a total of 5 times. The material bound to the Con A column was then eluted from the column by passing 7 ml elution buffer (Appendix 19) through the column; the eluate was collected as 7 x 1 ml fractions. All fractions were analysed by SDS-PAGE using the Phast system.

4. 2. 6. Immunisation regime:

Antibodies were raised in rabbits to the 42 and 52 k.Da antigens which had been separated by SDS-PAGE and eluted by electro-elution described in section 4.2.2. The eluates were emulsified in Freund's adjuvant at a ratio of antigen to adjuvant of 1:2 (v/v) using two 5 ml glass syringes connected through a luer fitting. The antigen solution was first injected into the adjuvant and the mixture was then passed repeatedly between the syringes until the emulsion became thick and creamy. The stability of the emulsion was tested by allowing a drop to fall onto the surface of a beaker of water. When the drop stayed intact the emulsion is considered ready for injection (Goding, 1986, Harlow and Lane, 1988). Two rabbits were immunised with each antigen. Each rabbit was injected intramuscularly (i.m) in a thigh muscle, using 25-gauge needle, with 100 μg of each antigen emulsified in Freund's Complete adjuvant (FCA). This was repeated 14 days later in the other hind limb. The

rabbits were then boosted three times by subcutaneous injection with 50 μg emulsified in Freund's Incomplete adjuvant (FIA) on days 49 and 77 and again with 25 μg of antigens in FIA on day 196. Five ml blood samples were collected on days -1, 24, 56, 84, 203 and a final 40 ml blood sample was collected on day 210 post primary injection for the preparation of serum.

Monitoring antibody production:

An antibody-ELISA was used to measure the amount of antibody in the sera collected from the four rabbits on days 24, 56, 84 and 203 post primary injection of the antigens. The assay was also used to determine the titre of the antibodies in the final serum collected on day 210 post-injection. Each serum was tested against a 1/80 dilution of a freeze-thawed soluble extract of the homologous *T. evansi* population as antigen. Sera were evaluated over a 2-fold dilution range from 1/250 to 1/8000 for the sera collected 24-203 days and over a 10-fold dilution range from $1/10^2$ to $1/10^6$ for the final serum collected at 210 days. In each case pre-immunisation serum at a similar dilution range was included as a negative control. All serum samples were tested in duplicate and the assay was performed as described in section 2.8. The antibody titre was taken as the last dilution that continued to show an absorbance of at least twice that of the negative control.

4. 2. 7. Specificity of the antibodies in day 210 sera:

Specificity and reactivity by antibody-ELISA:

The reactivity of the 210 day serum collected from rabbits immunised with either 42 or 52 k.Da antigens was examined by ELISA testing against freeze-thawed soluble extracts of homologous (TREU 2165) and heterologous

(TREU 2222 and 2257) *T. evansi* populations coated at a dilution of 1/80. Serum raised to the 52 k.Da antigen was also tested against freeze-thawed soluble extracts of *T. brucei* (TREU 2185) and *T. vivax* (TREU 2130) coated at a dilution of 1/80. All sera were tested at a dilution of 1/1000. Pre-immunisation serum at a dilution of 1/1000 and PBS/Tween wells were also included on each plate as controls. The assay was performed as described in section 2.8. and test samples showing optical density (O.D) values of greater than twice that of the pre-immunisation serum were considered as antibody positive to that antigen.

Specificity and reactivity by western immunoblotting:

This was used to examine the specificity and ability of the antibodies to react in this type of assay system. The antiserum raised to each antigen was tested by immunoblotting against whole trypanosomes extract, prepared by boiling in SDS sample buffer (section 3.2.3), from homologous (TREU 2165) and heterologous (TREU 2222 and 2257) *T. evansi* populations. 50 µl/well of the appropriate trypanosomes extract were subjected to SDS-PAGE (section 2.7) and electrophoretically transferred to nitrocellulose membrane as described in section 2.7.1. The membranes were incubated with blocking buffer (Appendix 7) for one hour at room temperature before incubation with appropriate serum samples diluted to 1/50 in blocking buffer. Unreacted serum was removed by washing as described in section 2.7.1. Peroxidase-labelled donkey anti-rabbit whole IgG molecule (Scottish Antibody Production Unit, Carluke, Lanarkshire) at a dilution of 1/500 in blocking buffer was then added to the membrane and any antigen-antibody binding was visualised by incubation with 4-chloro-1-α-naphthol substrate as described in section 2.7.1.

Reactivity by indirect fluorescent antibody test (IFAT):

This assay was used to provide information on the location of both the 42 and 52 k.Da antigens within *T. evansi* by testing the antisera against homologous (TREU 2165) and two heterologous (TREU 2222 and 2257) *T. evansi* populations. Parasitaemic whole blood obtained from mice infected with the appropriate *T. evansi* population was fixed either as smears in acetone (section 2.10.2.) or in suspension using formalin (section 2.10.1.) was used as antigen. In both cases reaction zones were marked on the microscope slides bearing the fixed trypanosomes as described in section 2.10.1.

For immunofluorescence testing 50 µl of each antiserum was diluted to 1/50 in PBS and added to the reaction zones marked on the microscope slide bearing the antigen. Serum collected on day 21 from rabbit infected with *T. evansi* (TREU 2165), pre-immunisation serum and PBS were included on each slide as positive and negative controls respectively. FITC-labelled donkey anti-rabbit IgG (Scottish Antibody Production Unit, Carluke, Lanarkshire) at a dilution of 1/40 was used as conjugate and the tests were performed as described in section 2.10.3.

Agglutination test:

The variant specificity and agglutinating properties of antibodies in each antiserum was examined by agglutination testing against homologous (TREU 2165) and heterologous (TREU 2222 and 2257) *T. evansi* populations. The serum was tested over a 2-fold dilution range from 1/2 to 1/1024 and the test was performed in micro well plate as described in section 2.9.

4. 3. RESULTS:

4. 3. 1. Preparative gel electrophoresis:

The eluate of the gel slices from both the 42 and 52 k.Da region from the gel showed a single band of the appropriate molecular size when subsequently analysed by SDS-PAGE. A similar amount of protein was obtained for each antigen following electro-elution from the 10 gels with 671 mg/ml protein for the 42 k.Da and 625μg/ml of the 52 k.Da protein after ultrafiltration concentration.

4. 3. 2. Chromatographic Purification Of Antigens:

The five fractions representing the major elution peak of the HPLC ion-exchange column (Figure 4. 1) showed multiple protein bands when analysed by SDS-PAGE Phast gel (Figure 4.2). Three to nine protein bands per fraction were detected by electrophoresis with molecular weights ranging from approximately 14 k.Da to 94 k.Da. The first four fractions included the 42 and 52 k.Da target proteins (Figure 4.2, lanes 2-5), while the fifth fraction included the 52 k.Da (Figure 4.2, lane 6).

After fractionation of the pooled ionex fractions 3-7 by size exclusion chromatography nine fractions were obtained that constituted the major elution peak (Figure 4. 3). After analysis by SDS-PAGE one to three protein bands per fraction were identified (Figure 4.4, lanes 4-10) with a molecular size of approximately 66, 87 and 94 k.Da.

When the major ion-exchange peak (pooled fractions 3-7) was subjected to lectin affinity chromatography using a Con A sepharose column, the first six fractions eluted from the column with elution buffer all contained

multiple bands when analysed by SDS-PAGE using the Phast system (Figure 4.5, lanes 2-7). Five protein bands were identified in each fraction with molecular weights of approximately 15, 30, 62, 66 and 94 k.Da. The seventh eluate fraction from this column (Figure 4.5, lane 8) did not contain any protein material resolvable by SDS-PAGE.

4. 3. 3. Antibody Response In The Immunised Rabbits:

Serum from test bleeds collected from the immunised rabbits 10 days following each injection of the antigen showed a progressive increase in absorbance values when tested by ELISA against a soluble extract of the homologous population (Figure 4. 6 and 4. 7). At a serum dilution of 1/250 a 3-fold increase in the absorbance value from day 24 to day 203 post-primary injection was observed. Anti-serum to the 42 k.Da antigen showed an absorbance value of 0.773 at day 24 which increased to 2.028 at day 203 (Figure 4.6). That of the 52 k.Da showed an absorbance value of 0.517 at day 24 which increased to 1.470 at day 203 post-primary injection (Figure 4.7).

The antibody titre of the final serum raised to the 42 k.Da antigen collected on day 210 post-immunisation was 1/100000 as defined by antibody-ELISA against the homologous soluble extract (Fig. 4. 8). The final serum to the 52 k.Da antigen had an antibody titre of 1/10000 when analysed by antibody-ELISA (Figure 4. 9).

4. 3. 4. Characterisation of the 210 day Anti-sera Raised To Each Antigen:

Anti-serum raised to the 42 k.Da antigen:

This serum was tested for reactivity against soluble extracts of the homologous and heterologous *T. evansi* populations using an ELISA test, and

reacted only with the homologous population of *T. evansi* giving an absorbance value of more than 9 x that of the pre-immunisation serum (Figure 4.10). In the case of the heterologous populations TREU 2222 and TREU 2257 absorbance values of less than twice that of the pre-immunisation serum were obtained when the serum was tested against the soluble extracts of these populations (Figure 4. 10).

Western immunoblotting of the whole cell extract with the antiserum raised to the 42 k.Da antigen recognised four faintly stained protein bands of molecular weight of approximately 65, 35, 29 and 26 k.Da and a strong 42 k.Da band in the homologous population of *T. evansi* (TREU 2165) (Fig. 4.11 lane No. 3). The serum did not recognise any antigenic components in the heterologous populations TREU 2222 and TREU 2257 (Figure 4. 12 lane No. 1A and 1B).

Fluorescent trypanosomes were detected in both formalin and acetone-fixed preparations of the homologous population (TREU 2165) when serum raised to the 42 k.Da antigen was tested against this population by IFAT. A strong green fluorescence was observed over the entire outline of both formalin and acetone-fixed trypanosomes. No fluorescent trypanosomes were detected with the heterologous populations TREU 2222 and TREU 2257 of *T. evansi*.

The serum to the 42 k.Da antigen did not agglutinate either homologous or heterologous *T. evansi* populations.

Anti-serum raised to the 52 k.Da antigen:

This anti-serum reacted with both the homologous (TREU 2165) and heterologous (TREU 2222 and 2257) populations of *T. evansi* as well as *T. brucei* and *T. vivax* populations (Fig. 4. 13) by ELISA. Each of the soluble trypanosome extracts showed absorbance levels greater than twice that of the

negative control serum. The *T. vivax* antigens showed the highest absorbance (1.028) followed by the *T. evansi* heterologous population TREU 2222 (0.717) and TREU 2257 (0.668). The homologous population of *T. evansi* (TREU 2165) showed an absorbance value of 0.630 and the absorbance value obtained with the *T. brucei* antigens was 0.481.

The serum recognised a single protein band of molecular weight of ~52 k.Da in the homologous population of *T. evansi* (TREU 2165) (Fig. 4.11 lane No. 2) by western immunoblotting. While in the heterologous population of *T. evansi* (TREU 2222 and 2257) two components were recognised - a major component with a molecular weight of approximately 52k.Da and a minor 33 k.Da component (Figure 4. 12 lane No. 2A and 2B).

No fluorescent trypanosomes were seen with either formalin or acetone-fixed parasites of the homologous (TREU 2165) or heterologous (TREU 2222 and 2257) *T. evansi* populations.

The serum did not agglutinate either homologous or heterologous *T. evansi* populations.

Figure 4. 2

Protein profile of the HPLC ion-exchange eluates: Silver staining on 8-25% gradient acrylamide Phast gel.

Lanes 1 and 7: Molecular weight markers.

Lanes 2 - 6: Fractions 3 to 7 representing the elution peak.

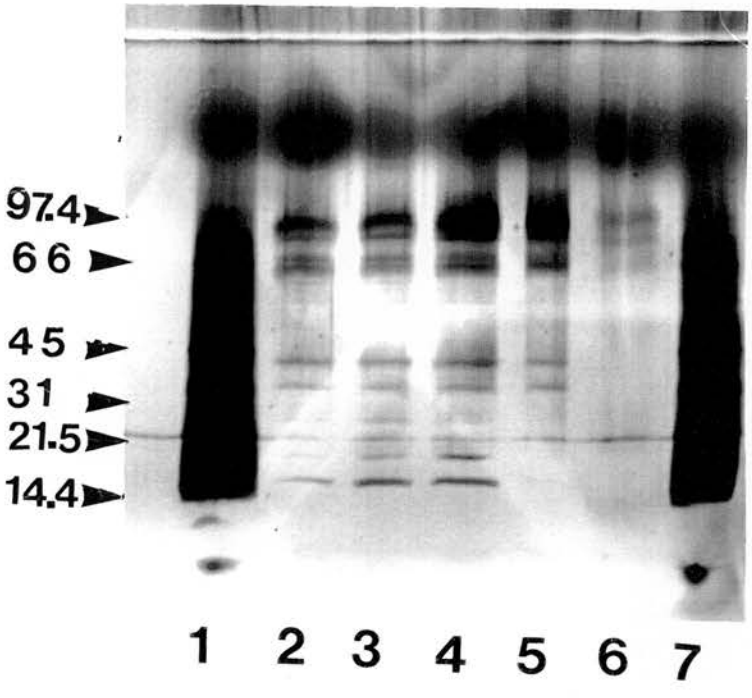

97.4
66
45
31
21.5
14.4
1 2 3 4 5 6 7

Figure 4. 3

Fractionation of *T. evansi* (TREU 2165) antigens by HPLC (size exclusion chromatography).

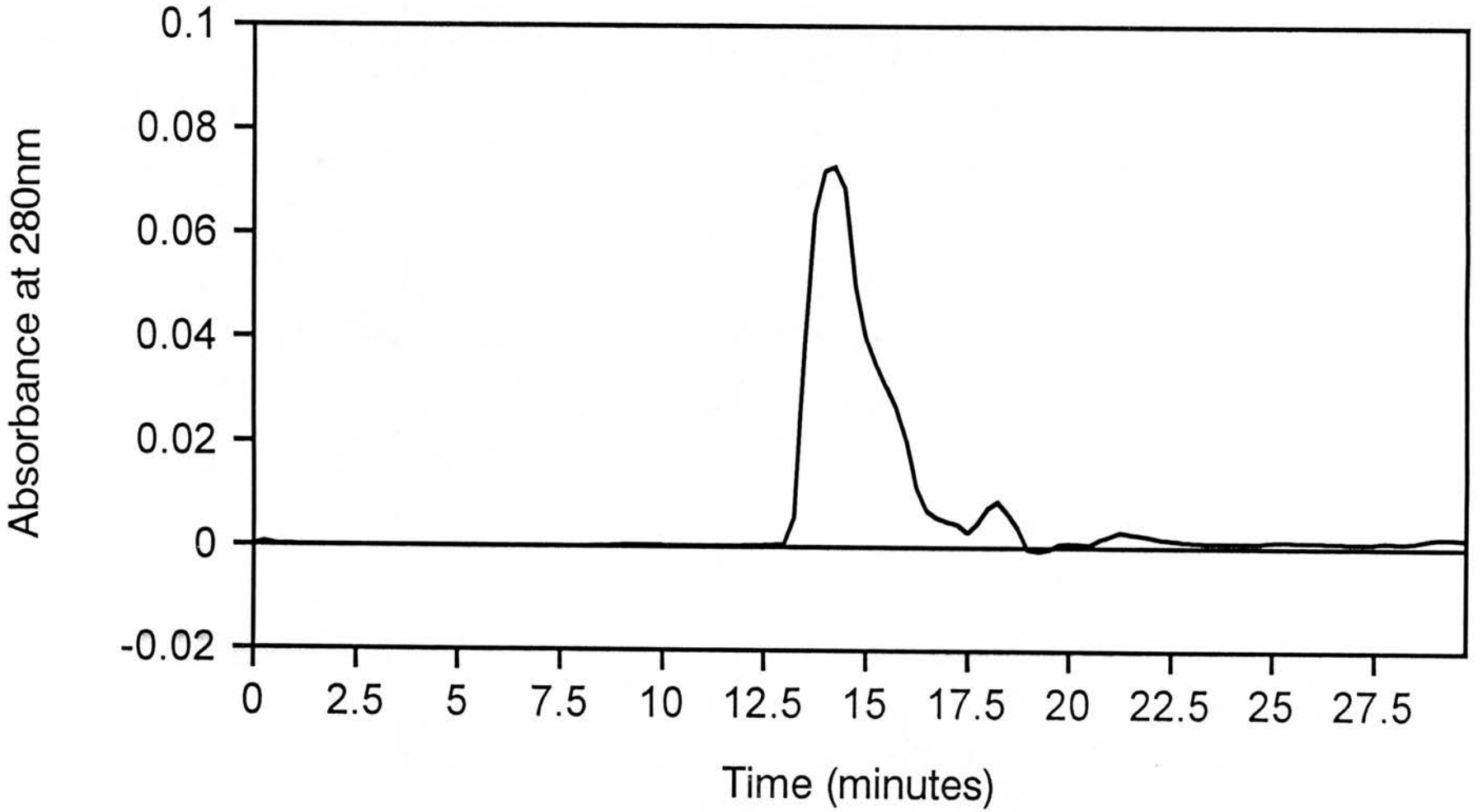

0.1
0.08
0.06
0.04
0.02
0
-0.02
Absorbance at 280nm
0
2.5
5
7.5
10
12.5
15
17.5
20
22.5
25
27.5
Time (minutes)

Figure 4. 4

Protein profile of fractions separated by HPLC size exclusion column. Silver staining on 15% acrylamide mini-gel.

Lane 1 : Molecular weight markers.

Lanes 2-10: Fractions 12-20 representing the elution peak.

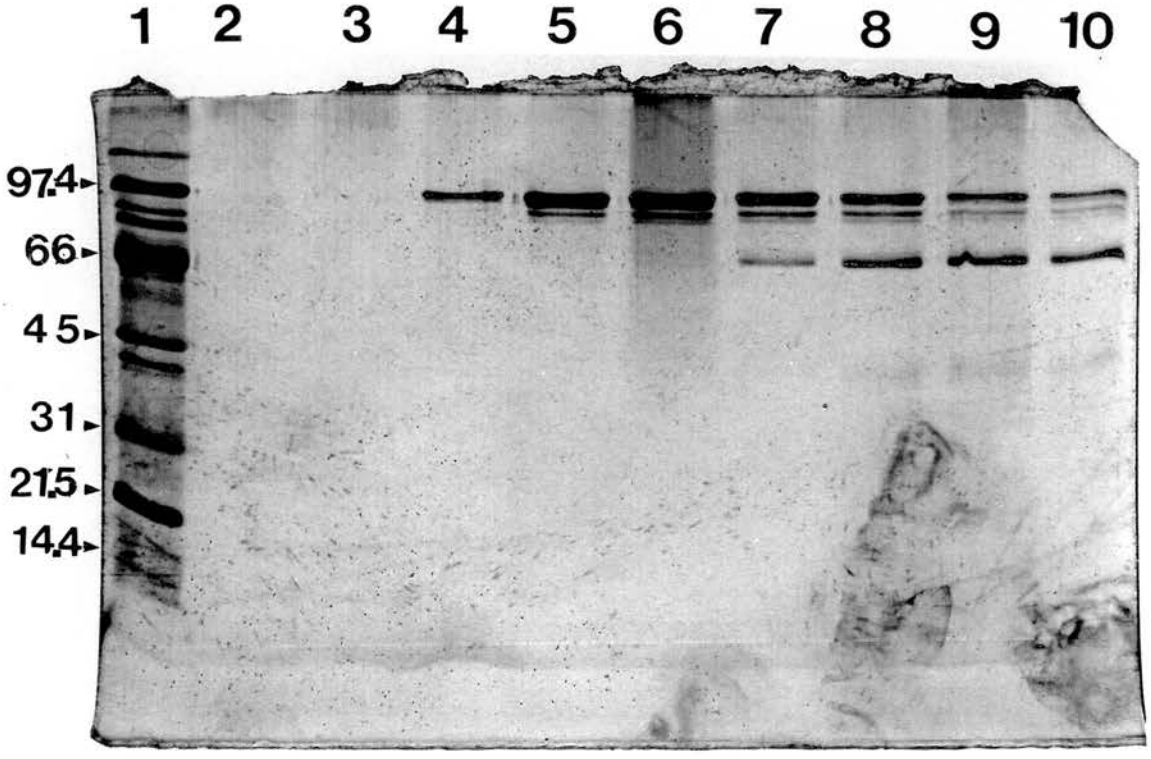

1 2 3 4 5 6 7 8 9 10
97.4
66
45
31
21.5
14.4

Figure 4. 5

Protein profile of Concanavalin A column eluates: Silver staining on 8-25% gradient acrylamide Phast gel.

Lane 1: Molecular weight markers.

Lanes 2-8: Fractions 1-7 from the column eluate.

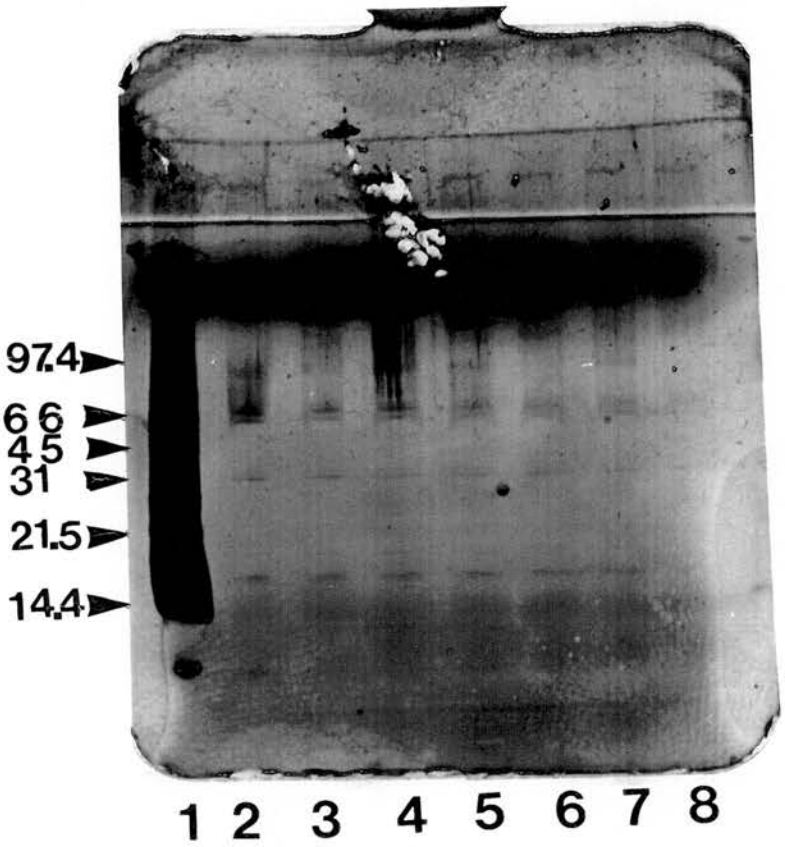

97.4
66
45
31
21.5
14.4
1 2 3 4 5 6 7 8

Figure 4. 6

Antibody response in rabbits immunised with the 42 k.Da *T. evansi* antigen. Antibody-ELISA against the homologous *T. evansi* population.

Days 49, 77 and 196 indicate the immunisation boosts.

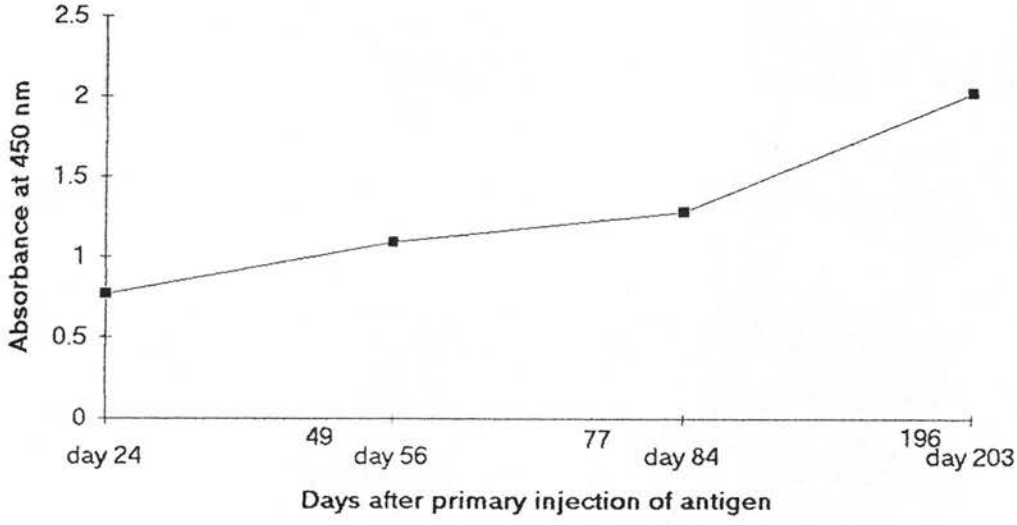

2.5
2
1.5
1
0.5
0
Absorbance at 450 nm
day 24
49
day 56
77
day 84
196
day 203
Days after primary injection of antigen

Figure 4. 7

Antibody response in rabbits immunised with the 52 k.Da *T. evansi* antigen. Antibody-ELISA against the homologous *T. evansi* population.

Days 49, 77 and 196 indicate the immunisation boosts.

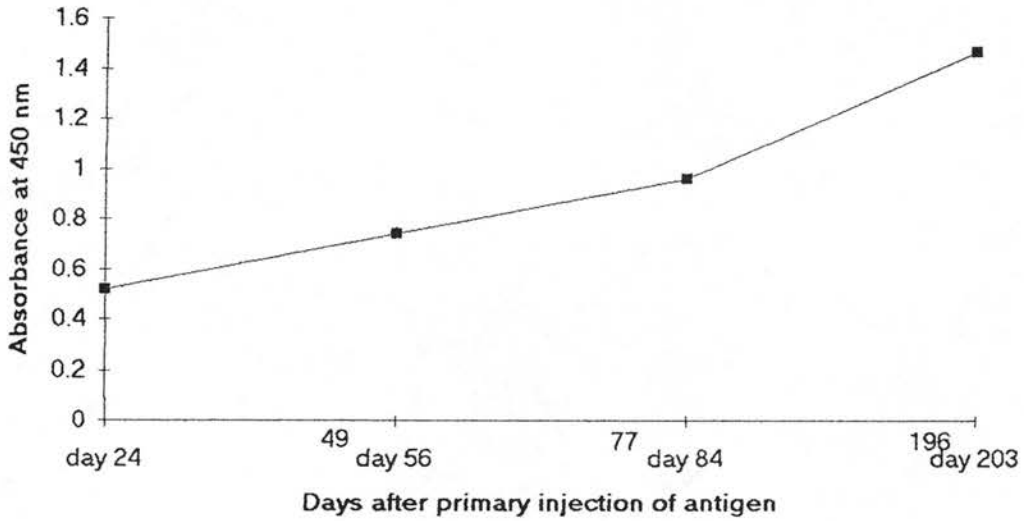

1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Absorbance at 450 nm
day 24
49
day 56
77
day 84
196
day 203
Days after primary injection of antigen

Figure 4. 8

Titration of antibodies in the final serum collected from the rabbits immunised with the 42 k.Da antigen by ELISA against soluble extract of homologous trypanosome population.

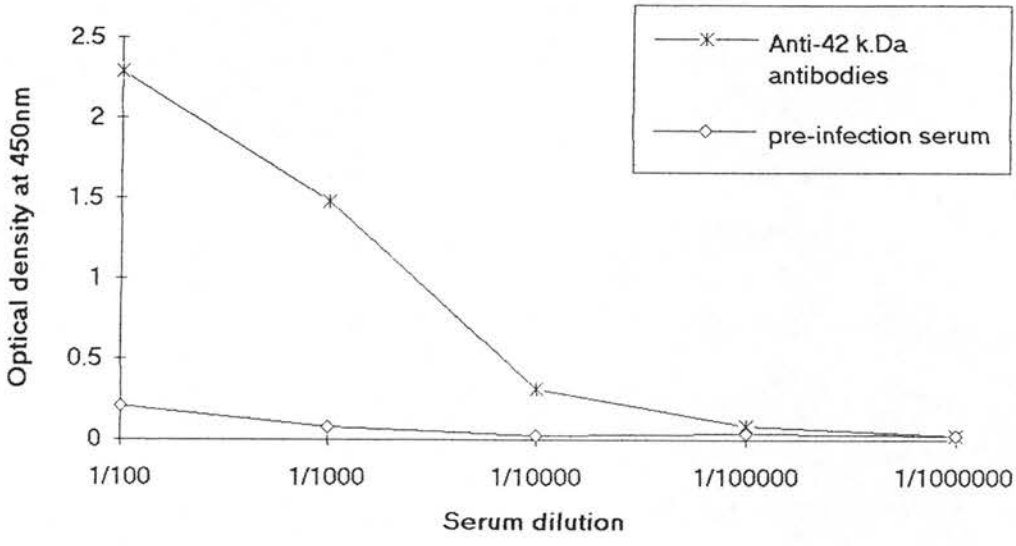

Anti-42 k.Da antibodies
pre-infection serum
Optical density at 450nm
2.5
2
1.5
1
0.5
0
1/100
1/1000
1/10000
1/100000
1/1000000
Serum dilution

Figure 4. 9

Titration of antibodies in the final serum collected from the rabbits immunised with the 52 k.Da antigen by ELISA against soluble extract of homologous trypanosome population.

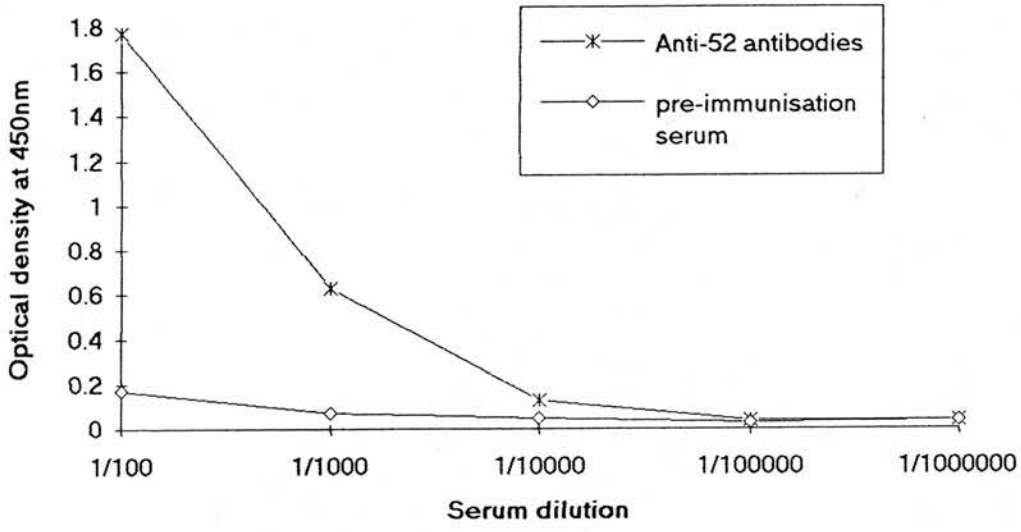

Anti-52 antibodies
pre-immunisation serum
Optical density at 450nm
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
1/100
1/1000
1/10000
1/100000
1/1000000
Serum dilution

Figure 4. 10

Reactivity of anti-serum to the 42 k.Da antigen with homologous and heterologous-T.*evansi* populations by ELISA.

TR2165: *T. evansi* homologous population (TREU 2165).

TR2222 & TR2257: *T. evansi* heterologous populations (TREU 2222 & 2257).

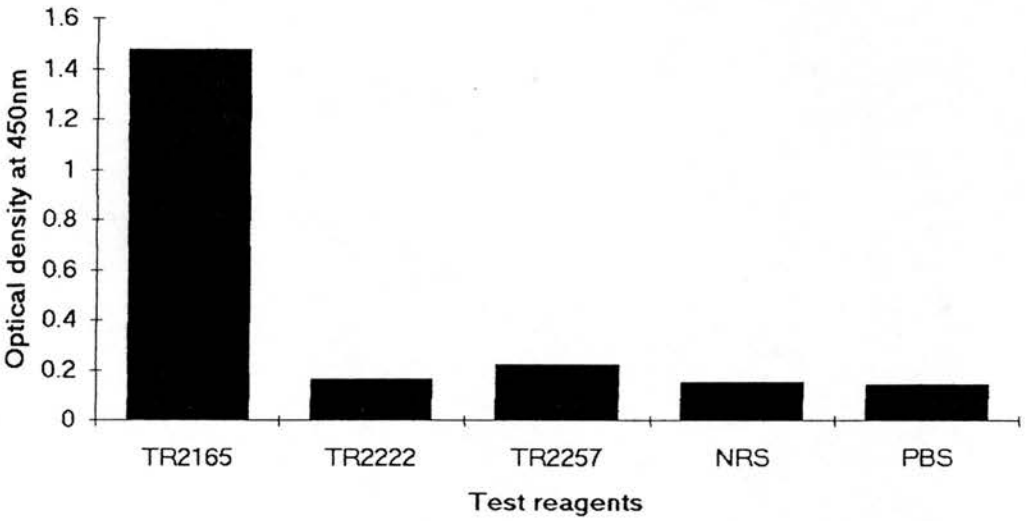

Optical density at 450nm
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
TR2165
TR2222
TR2257
NRS
PBS
Test reagents

Figure 4. 11

Results of western immunoblotting of homologous *T. evansi* population (TREU 2165) against sera to the 42 and 52 k.Da antigens.

Lane 1: Molecular weight markers.

Lane 2: Antiserum to 52 k.Da antigen.

Lane 3: Antiserum to 42 k.Da antigen.

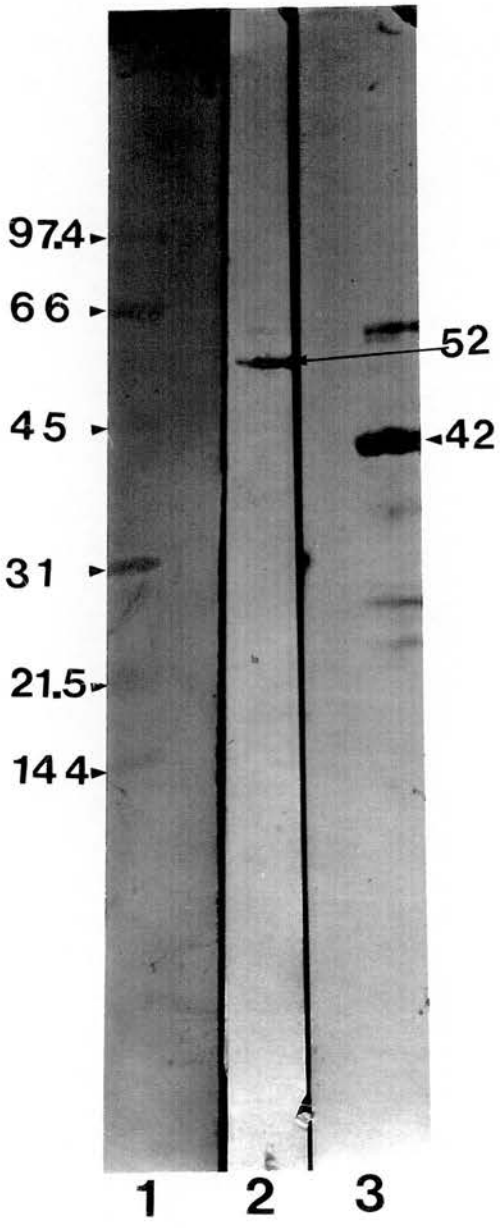

97.4
66
45
31
21.5
14.4
52
42
1
2
3

Figure 4. 12

Results of western immunoblotting of heterologous *T. evansi* population (TREU 2222 and 2257) against sera to the 42 and 52 k.Da antigens.

Lane 1: Antiserum to 42 k.Da antigen.

Lane 2: Antiserum to 52 k.Da antigen.

Lane 3: Molecular weight markers.

a ≡ *T. evansi* TREU 2222.

b ≡ *T. evansi* TREU 2257.

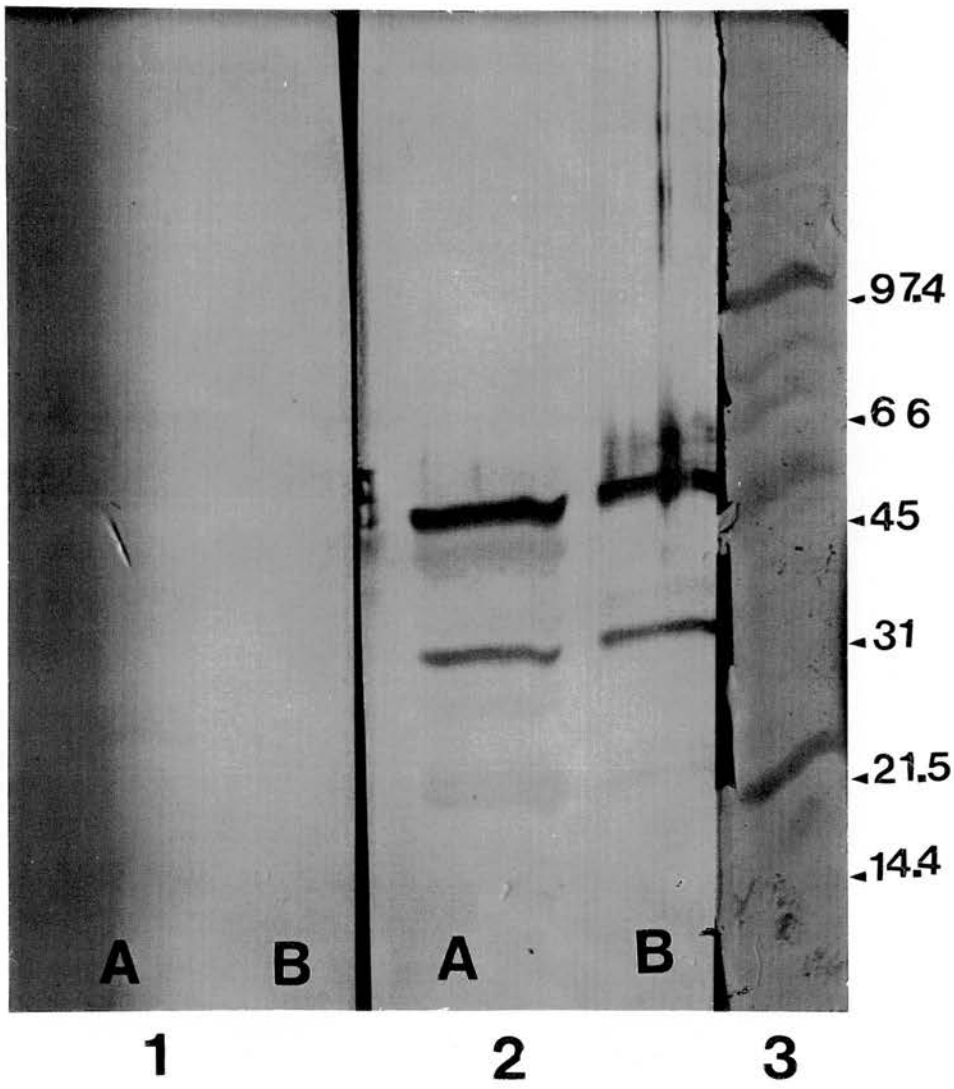
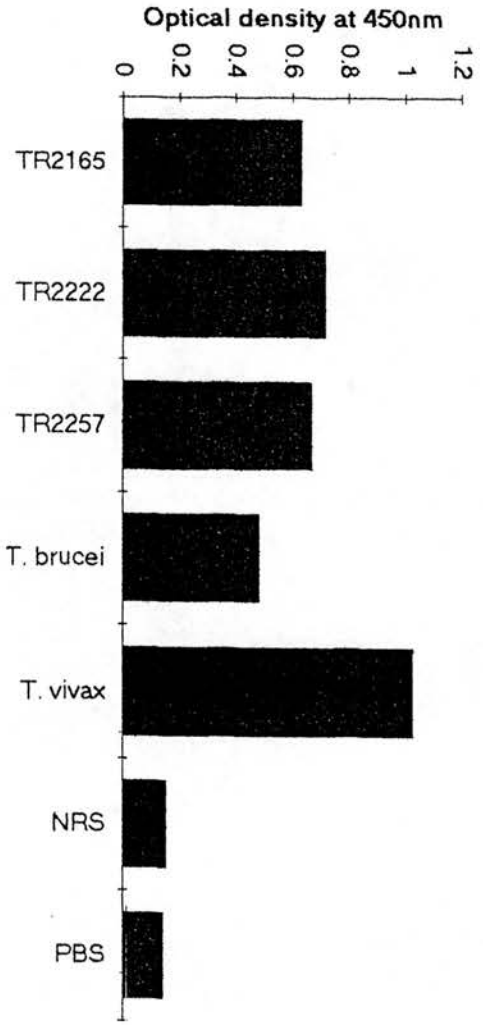

97.4
66
45
31
21.5
14.4
A
B
A
B
1
2
3

Figure 4. 13

Reactivity of antiserum to the 52 k.Da antigen by ELISA against homologous and heterologous *T. evansi* populations, and *T. brucei* and *T. vivax*.

TR2165: *T. evansi* homologous population (TREU 2165).

TR2222 & TR2257: *T. evansi* heterologous populations (TREU 2222 & 2257).


Optical density at 450nm
0
0.2
0.4
0.6
0.8
1
1.2
TR2165
TR2222
TR2257
T. brucei
T. vivax
NRS
PBS

4. 4. DISCUSSION:

Immunisation of rabbits with the *T. evansi* antigens eluted from Coomassie stained polyacrylamide gels in the present study elicited strong antibody responses to the 52 k.Da and 42 k.Da antigens by immunoblotting and ELISA. This result indicates that both antigens retained their immunogenicity and confirms the usefulness of the SDS-PAGE for the purification of antigens for immunisation as reported by previous workers (Stumph *et al*, 1974, Harlow and Lane, 1988).

The 52 k.Da antigen was found to be conserved between different populations and stocks of *T. evansi* as well as across species in *T. brucei* and *T. vivax*. Anti-serum raised against this antigen in the present study recognised antigens in a heterologous *T. evansi* population of the same stock (TREU 2222) and a different stock (TREU 2257) as well as in stocks of *T. brucei* and *T. vivax* . Immunoblotting with the serum recognised the target antigen as a single component in the homologous population but as two components of 52 and 33 k.Da in the heterologous *T. evansi* population. This 33 k.Da component might be a breakdown product or another component only present in the hetereologous population carrying similar epitopes to the 52 k.Da

These findings indicate that the 52 k.Da represents an invariant antigen as invariant antigens of molecular size ranging from 20 to 300 k.Da had been reported from different species and populations of trypanosomes. Some of these invariant antigens have been shown to be located on the surface of the parasite (reviewed by Overath *et al*, 1994), while others are non-surface associated antigens (Shapiro and Murray, 1982, Stieger and Seebeck, 1986, Frommel and Balber, 1987, Muller *et al*, 1992). Evidence from the present study suggests that the 52 k.Da antigen is not surface-associated as serum raised to it did not react by IFAT or agglutination. Although IFAT can detect

both surface and non-surface antigens, it prossibly biased towards surface antigens as formalin fixation is reputed to preserve the surface coat antigenicity (Nantulya and Doyle, 1977).

The 42 k.Da antigen in contrast to the 52 k.Da appeared to be a population-specific antigen as serum raised against this antigen only recognised antigenic materials in the homologous *T. evansi* population (TREU 2165) by immunoblotting, ELISA and IFAT. Results from immunoblotting, however, showed that the serum recognised five protein bands in the homologous population of the parasite although dominated by the 42 k.Da protein. Such multiple recognition could be due to contamination of the original gel slice or breakdown products arising from protease activity during preparation of extracts. Contamination from the nearby bands during excision of the antigen from gels is unlikely since the contaminants have large molecular size range. The 65 k.Da contaminant is clearly not a breakdown product of the 42 k.Da although the 42k.Da component might be a breakdown product of the 65 K.Da component in the extracts used for SDS-PAGE. Similarly, the other components could be breakdown product of either the 65 or 42 k.Da components.

One of the heterologous populations of *T. evansi* used in the present study was derived from the same stock as the homologous population (section 2.1) and is known to be antigenically different from the homologous population (T. W. Jones, personal communication). This suggests that the 42 k.Da antigen complex is variant-specific although the molecular weight all but the 65K.Da component is lower than that reported for *T. evansi* VSG (65-67 k.Da, Uche, 1989).. Although there is evidence that the 42 k.Da is surface-associated from the overall green fluorescence seen by IFAT, the anti-serum to this antigen did not agglutinate the homologous population of the parasite. This suggests that if

surface-associated, the 42 k.Da is not present in enough sites to cross-link for agglutination formation. It is possible, however,that drying and fixation of the trypanosomes during antigen preparation could have exposed the antigen to the serum in IFAT making it appear to be surface-associated.

Results (not presented) from densitometric measurements on Coomassie Blue-stained SDS-PAGE gels indicate that the 42k.Da antigen is present in greater amount in the parasite materials than the 52 k.Da). This might also explain the reaction of its antiserum in IFAT. Another explanation of the reaction of the anti-42 k.Da serum in IFAT is that the antigen is possibly sharing an identical epitope with the surface coat antigen. This hypothesis will also explain the importance of the variant-specificity of the 42 k.Da antigen, as after switching from one VSG to another the previous VSG will be only be gradually diluted during succeeding divisions (Overath et al, 1994) and the presence of the 42 k.Da will possibly lower the chances of VSG-specific antibodies to bind to the surface of the trypanosomes, thereby, allowing the switching process to succeed.

None of the HPLC chromatographic procedures produced either the 42 or 52 k.Da antigens from *T. evansi* lysates in sufficient purity for further study. Other systems such as reverse-phase high-performance liquid chromatography might have produced better separation of these parasite antigens and have been used for the purification of *T. brucei* VSG (Clarke *et al*, 1984). The organic solvents used with this method however, might degrade the proteins reducing their immunogenicity.

Despite the overall failure of the HPLC to separate the antigens, it did provide additional information on the characteristics of these two antigens.

1. The isoelectric point (pI).of both antigens is probably close to 7.0 as they were eluted during the early stages of ion-exchange chromatography indicating that they were not tightly bound to the column at pH 7.0
2. The smallest component resolved by size exclusion chromatography was 66k.Da. This would be in keeping with the failure to isolate either the 42 or the 52 k.Da antigens by this method under these conditions.
3. Neither 42 nor the 52 k.Da antigens possesed any of the sugar residues that are known to bind Con A such as α-linked D-mannosyl, D-glucosyl and N-acetyl-D-glucosaminoyl (Lis and Sharon, 1973, Rautenberg *et al*, 1980, Frommel and Balber, 1987) as neither bound to Concanavalin A.

The present study has, therefore,confirmed the usefulness of SDS-PAGE separated proteins as a mean of producing highly-specific, high titred antisera to individual trypanosomes proteins and highlighted the problems associated with chromatographic separation of complex mixtures of proteins with similar physico-chemical properties.. The antibodies produced in this part of the study will be used to develop immunoassays for the detection of the two antigens in serum and tissues of infected animals.

CHAPTER FIVE

PRODUCTION OF MONOCLONAL ANTIBODIES TO *TRYPANOSOMA EVANSI* (TREU 2165)

5. 1. AIM:

To develop monoclonal antibodies (McAbs) to *T. evansi* antigens for use in detecting antigens in the circulation and tissues of infected animals.

5. 2. INTRODUCTION:

Monoclonal antibodies (McAbs) are highly selective reagents that can be used in the identification and isolation of individual antigens from trypanosomes. Unlike polyclonal mono-specific antibodies, a pure antigen is not required for immunisation of animals for monoclonal antibody production due to cloning and selection during production.

The introduction of a cell fusion technique by Kohler and Milstein (1975) enabled the routine growth of populations of antibody-secreting cells in *vitro*. In this technique an *in vivo* produced antibody-secreting cell is fused with a myeloma cell to produce a hybridoma which can then multiply in an appropriate tissue culture system. Such hybridomas can produce unlimited quantities of antibodies with defined specificity. Hybridomas can also be propagated in *vivo* in histocompatible animals and produce ascites fluid containing up to 5-20 mg/ml of antibodies (Goding, 1986, Liddell and Cryer, 1991).

As with polyclonal antibody production, the immunisation regime used in McAb work greatly influences the type of response in the animals from which antibody-secreting cells will be isolated. Various immunisation methods have been successfully used for the production of McAbs to trypanosomes, the

majority of which have been directed at variant-specific surface antigens. A common approach in these methods involves infection of the host followed by drug-treatment to arrest the development of new populations of trypanosomes and to prevent the infected animal dying. Purified VSGs have been used in immunisation regimes for production of McAbs (Myler *et al*, 1985; Masterson *et al*, 1988). Unfortunately McAbs to purified VSGs do not usually recognise the VSG molecule on living trypanosomes probably due to alteration of the antigen during the initial purification process. The duration of the immunisation protocol determines the class of antibody produced. IgM antibodies are usually produced with a short period of immunisation and a single infection (Theodos *et al*, 1990). IgG antibodies are generally produced by longer immunisation protocols involving multiple inoculations of living trypanosomes (Masterson *et al*, 1988) and boosting with soluble trypanosome extracts (Theodos *et al*, 1990, Alves *et al*, 1983).

Similar approaches employing multiple inoculations with living trypanosomes have been used to produce McAbs to invariant surface antigens (Burgess and Jerrells, 1985). Infection and boosting protocols have also been used to produce McAbs to non-surface antigens such as the cell membrane of *T. congolense*, *T. vivax* (Nantulya *et al*, 1987) and *T. brucei* (Nantulya *et al*, 1987, Colmerauer *et al*, 1989, Turner *et al*, 1989) procyclic trypanosomes. As in the case of VSG, purified non-surface antigens were used for McAbs production (Stieger and Seebeck, 1986, Gallo and Schrevel, 1985).

5. 3. DETERMINATION OF IMMUNISATION PROTOCOLS:

Two immunisation protocols previously used to produce McAbs to trypanosomes were compared in this pilot study; a short immunisation protocol as described by Theodos *et al* (1990) and a long protocol as described by Masterson *et al*, (1988).

5. 3. 1. Materials and Methods:

Trypanosomes

The history of *T. evansi* TREU 2165 the parasite stock used in this study is presented in section 2. 1.

Immunisation protocols:

Two groups of 5 mice were used for this study. One group (A) was infected i.p. with $2.6x10^6$ *T. evansi* trypanosomes per mouse and treated at a dose rate of 335 μg Berenil / mouse for 3 consecutive days starting three days post-infection according to Theodos *et al* (1990). The mice in the second group (B) were infected as above but treated two days later with a single dose of 5 mg Berenil / Kg body weight. Group B was boosted 30 days post-infection with $2.6x10^6$ trypanosomes per mouse and was then treated on the next day with Berenil at a dose rate of 5 mg/kg.

For individual mice in the two groups 100 µl tail blood was collected for serum on days 4, 6, 10, 17, 23, 37, 42 and 45 post infection and tested for antibody.

Monitoring antibody response:

Indirect fluorescent antibody test (IFAT): Living trypanosomes as antigen:

Sera collected from both groups of mice on days 4, 6, 10, 17, 23, 37, 42 and 45 were tested by IFAT for antibody response to surface antigens using live trypanosomes as antigen as described by Doyle *et al* (1980). Column separated trypanosomes (section 2.3) were resuspended in PSG to a concentration of 1 x 10^7/ml. 100 µl of the trypanosome suspension was incubated in a microcentrifuge tube for 30 minutes at 4°C with an equal volume of mouse serum previously diluted to 1/10 in PBS. The trypanosomes were then washed 3 times in PSG by centrifugation at 3000 r.p.m. for 5 minutes at 4°C and resuspended in 100 µl of fluorescein isothiocyanate (FITC)-labelled sheep anti-mouse IgM or IgG (The Binding Site Ltd, Birmingham, U.K) diluted to 1/20 in PBS. After incubation for 30 minutes at 4°C the trypanosomes were washed as before and then resuspended in 10 µl of tris-buffered glycerol, pH 9.5, containing 2% (v/v) gluteraldehyde. Each preparation was mounted under a cover-slip on a microscope slide and examined by combination of phase-contrast and incident fluorescence microscopy using a Diaplan fluorescence microscope (see section 2.10.3). At least 50 parasites were examined per sample and the number of fluorescent trypanosomes recorded as a percentage of the total number of trypanosomes.

IFAT using formalin-fixed trypanosomes:

Immunofluorescence utilising formalin-fixed, air-dried *T. evansi* (TREU 2165) trypanosomes was used to monitor the IgG response to the parasite in the group B mice following challenge with 2.6 x 10^6 homologous trypanosomes. Serum collected from group B mice on days 37, 42 and 45 post-infection diluted 1/10 in PBS, and FITC-labelled sheep anti-mouse IgG diluted 1/20 in PBS were used in this test. Details of the test are described in section 2.10.3.

Agglutination test:

Serum collected from both groups of mice on days 4, 6, 10 and 23 post-infection was tested for agglutinating antibodies over a 2-fold dilution range from 1/2 to 1/1024 by incubation with the homologous *T. evansi* population (TREU 2165). Details of the test are described in section 2.9.

5. 3. 2. RESULTS:

Antibody response in mice of the short immunisation protocol (group a):

The anti-IgM conjugate labelled 16% of the live trypanosomes 4 days after infection, with a peak labelling of 85% at day 6 but fell to an undetectable level by 37 days after infection (Table 5.1).

The anti-IgG conjugate did not label any trypanosomes until 17 days after infection when 75% of the living trypanosomes were labelled. This reached 100% labelling 6 days later with a slight fall thereafter but maintained a high level up to day 45 after infection (Table 5.1).

Agglutinating antibody levels in the immunised mice were similar in pattern to IgM levels with peak levels occuring at day 10. Levels of agglutinating antibodies fell to 1/64 by day 23 after infection (Table 5.1).

Antibody response in Group B mice:

Using living trypanosomes as IFAT antigen, the IgM response in this group was similar to that seen in group A. The peak activity of anti-IgM conjugate was detected on day 6 then gradually fell to undetectable levels 37 days after infection (Table 5.2).

The action of IgG conjugate was detected slightly earlier in this group by day 10 after infection. The activity increased reaching 100% labelling of the living trypanosomes by 42 days a level that was maintained until the end of the experiment on day 45(Table 5. 2).

When formalin-fixed trypanosomes were used as the IFAT antigen 100% labelling was detected with all sera collected from group B following challenge with trypanosomes on day 30 (Table 5.2).

Agglutinating antibodies in group B were not detected before day 6 post-infection, but a higher level of these antibodies was maintained compared to group A animals (Table 5.2).

Table 5. 1

The percentages of labelled trypanosomes by the anti-IgM and IgG conjugates and the agglutination antibodies in the group A mice.

Days after infection	Percentage of tryps labelled by anti-IgM	Agglutinating antibodies titre	Percentage of tryps labelled by anti-IgG
4	16.4	1/32	0
6	85.5	1/128	0
10	78.2	1/256	0
17	45	-	75
23	40	1/64	100
37	0	-	90
42	0	-	92
45	0	-	98

Table 5. 2

The percentages of labelled trypanosomes by the anti-IgM and IgG conjugates and the agglutination antibodies in the second group of mice (B) infected with *T. evansi*, treated with 5 mg Berenil/kg body weight two days later, rechallenged 30 days post-infection and treated on day 31.

Days after infection	% of tryps labelled by anti-IgM conjugate	Agglutinating antibodies titre	% of tryps labelled by anti-IgG	Formalin fixed trypanosomes
4	20	0	0	-
6	95.5	1/512	0	-
10	90.9	1/1024	9.1	-
17	50	-	70.5	-
23	40	1/1024	85	-
37	0	-	92	100
42	0	-	100	100
45	0	-	100	100

5. 3. 3. DISCUSSION:

Both immunisation protocols produced IgM and IgG responses in the mice. Similar patterns of antibody developed in both groups A and B mice, an initial IgM response which was later replaced by an IgG response. The overall pattern of the IgM response was, similar in both immunisation protocols in that it was first detected on day four, reached a peak level on day 6 and declined thereafter until it disappeared on day 37 post-infection. The gradual increase in the number of labelled trypanosomes could either be attributed to the differences in antigenicity of the trypanosomes used in the test or more likely to a gradual maturation of epitope recognition by these antibodies since new variable antigen types (VAT) usually arise at 4 to 5 days intervals (Gray, 1965) and the trypanosomes used in the present test were grown in mice for three days only.

Little difference was observed in the pattern of the IgG response elicited by both immunisation protocols. The IgG response in group A mice was detected later than in group B and reached a peak on day 23 post-infection. Group B mice showed a steady increase in the level of the IgG over the course of the experiment. The challenge of group B on day 30 post-infection appears to have boosted the IgG in this group as would be expected . following a challenge with the same antigen (Harlow and Lane, 1988).

Formalin-fixed trypanosomes were compared with living trypanosomes as a source of antigen in the IFAT test. With live trypanosomes only the surface antigens are exposed to the serum in the test. Although formalin

fixation stabilises surface coat antigens while preserving their antigenicity (Nantulya and Doyle, 1977), non-surface antigens will also be exposed following drying and fixation of the parasites. A slight difference was observed in the present study in the percentage of labelled trypanosomes by the anti-IgG conjugate with 100% labelling observed on the formalin-fixed trypanosomes. For subsequent studies IFAT using formalin-fixed trypanosomes as an antigen source was chosen for screening the hybridomas for McAbs production, since it should be able to detect both surface and non-surface trypanosome antigens. It also has the additional advantage that fresh trypanosomes are not needed for performing the test, and that fixed trypanosomes can be kept for long periods without affecting the antigenicity of the trypanosome components (Nantulya and Doyle, 1977). The latter feature is an important criteria when large numbers of hybrid supernatant have to be tested as occurs in post-fusion screening.

For further studies the immunisation protocol used for the mice in group B was selected for the production of monoclonal antibodies to *T. evansi*. This choice was based on a number of criteria. First the IgG response was elicited earlier by this group than by the mice in group A. Second challenging with trypanosomes will activate more B lymphocytes which will improve the efficiency of the fusion of spleen cells with myeloma cells. Fusion usually requires recently activated B cells (Goding, 1980) and fusion is recommended on day 3 after the final immunisation (Oi *et al*, 1978). To bias class-specific antibodies, IgG antibodies were chosen because they contain high level of specific antibodies and are of high affinity to the immunogen (Harlow and Lane, 1988) an important criteria to consider when the antibodies are to be used in immunoassays.

Agglutination tests were carried out in this study to confirm the IgM response since these antibodies are known to be efficient agglutinators compared to IgG antibodies (Bellanti, 1985). Results obtained with this test generally confirm those of the IgM response.

The immunoglobulin response reported in this study is similar to those reported in other hosts for trypanosomes infection. The persistence of IgM antibodies up to 3 weeks post-infection as indicated by the level of agglutinating antibodies is similar to the findings of Seed *et al* (1969) and Zahalsky and Weinberg (1976) who reported a predominantly IgM response during the period of primary infection in rabbits infected with *T. b. gambiense* and rats or cattle infected with *T. b. brucei*. The predominantly high level of IgG antibodies from 17 days post-infection compared to the level of IgM antibodies occured regardless of the second challenge with trypanosomes and is in agreement with the predominant IgG response during primary infection reported by Campbell *et al* (1978), Nantulya *et al* (1979) and Uche (1989), for *T. b. rhodesiense*, *T. b. brucei* and *T. evansi* infections respectively.

5. 4. MONOCLONAL ANTIBODIES TO *T. EVANSI* (TREU 2165)

5. 4. 1. Materials and Methods:

Experimental animals:

Balb/C mice (Bantin and Kingman, U.K.), 6-8 weeks old, were used as source of sensitised lymphocytes and for ascites production.

Trypanosomes:

A *T. evansi* population (TREU 2165) was used for the immunisation of the Balb/C mice. Two other populations of *T. evansi* (TREU No's 2222 and

2257) representing two stocks of the parasite were used to characterise the antibodies. The history of these trypanosome populations is presented in section 2.1.

Immunisation protocol:

Cryopreserved *T. evansi* (TREU 2165) trypanosomes were expanded in cyclophosphamide treated mice (300 mg/Kg body weight, 24 hours before infection) and separated from the host blood using DE52 column chromatography, washed by centrifugation with PSG and counted by haemocytometry. The immunisation protocol described in section 5.3 was used with 4 Balb/C mice injected i.p. with $5x10^6$ trypanosomes per mouse and treated i.p. two days later with a single dose of 5 mg Berenil / Kg body weight. The mice were then boosted with the same number of trypanosomes on day 30 post-infection and then treated again with Berenil at a dose rate of 5 mg / Kg one day later. Tail blood was collected from the mice for serum on day 32. Two days later the spleen was removed from the mouse showing the highest level of antibodies and used to prepare cells for the first fusion. The remaining mice were boosted with $5x10^6$ trypanosomes on day 50 and treated with Berenil at a dose rate of 5 mg / Kg body weight one day later. Tail blood was collected from these mice for serum on day 52 and examined for antibody by ELISA. Two days later the spleen was removed from the mouse showing the highest level of antibodies and used to prepare cells for a second fusion.

Monoclonal antibody production:

Preparation of myeloma cells:

Mouse myeloma cell line NSO/1, a non-immunoglobulin-producing cell line (Galfre and Milstein, 1981), was resuscitated from storage in liquid nitrogen ten days prior to the day of fusion. The myeloma cells were passaged

in complete medium (Appendix 20) in 75 and 150 cm^2 tissue culture flasks (Gibco) at logarithmic phase of growth by subculturing every 48 hours. The myeloma cells were harvested for fusion by washing once in RPMI 1640 without FCS (in incomplete medium) at 1000 r.p.m. for 5 minutes and resuspension in 10 ml fresh incomplete medium. The viability of the cells was estimated by counting after staining with 0.2% trypan blue (Appendix 23).

Preparation of feeder cell layer:

An uninfected Balb/C mouse was killed by cervical dislocation. The spleen was removed in an aseptic manner, cut into small fragments and a cell suspension made by squashing the tissues with a blunt edge of a forsceps in a coarse sieve over a Petri-dish containing about 5 ml incomplete medium. The suspension was then frequently pipetted with sterile plastic pipette to disrupt the clumps of the spleen cells before 15 ml incomplete medium was added and the suspension transferred to a 50 ml centrifuge tube. After standing for several minutes the suspension was centrifuged at 1000 r.p.m. for 5 minutes. The pellet was suspended in 7 ml HAT medium (Appendix 21) containing 100 units/mg per ml of penicillin/streptomycin (Appendix 22) before counting the number of cells in an aliquot diluted in white blood cell counting fluid (Appendix 23). The cells were taken for irradiation for 98 seconds with 1500 rads using Caesium 137 to arrest cell growth. The irradiated suspension was made up to 36 ml with HAT medium and the cells pipetted out into 6 x 96 well flat bottomed microtitre plates (Gibco) with each well receiving 100 µl. Sixty wells were used per plate to minimise accidental loss and the plates were incubated in a humidified 37°C incubator in 5% CO_2 in air.

Preparation of mouse spleen cells for fusion:

One of the immunised Balb/C mice was killed by cervical dislocation. A spleen cell suspension was prepared as described previously and centrifuged at 1000 r.p.m. for 5 minutes. The pellet was then washed once in incomplete medium before suspending in 10 ml fresh incomplete medium and the number of cells counted after dilution in white blood cell counting fluid (Appendix 23).

Fusion of myeloma and spleen cells:

Myeloma and spleen cells were mixed together in a conical 50 ml tube at a ratio of 1:10 and centrifuged at 1000 r.p.m. for 5 minutes. The supernatant was poured off and a pipette used to remove the last few drops of the remaining supernatant. Over a period of 3 minutes, 1 ml of pre-warmed (37°C) polyethylene glycol (PEG, Boehringer) was added to the combined cell pellet with mixing between drops, and the final suspension was then incubated at 37°C for 2 minutes. The PEG enriched mixture was then diluted slowly by adding 1 ml, 5 ml and then 10 ml of incomplete medium dropwise over a period of 7 minutes while mixing by shaking the tube continuously. The suspension was centrifuged at 900 r.p.m. for 5 minutes, supernatant discarded and the pellet suspended in 36 ml HAT medium. The cells were then pipetted out into the plates previously coated with feeder cells, each well receiving 100 µl of cell suspension, and the plates were incubated in a humidified 37°C incubator in 5% CO_2 in air.

Post fusion protocol and initial antibody screening:

Seven days after the fusion, the microtitre plates were first examined for evidence of contamination. Wells showing turbidity and yellow medium colour were emptied and rinsed four times with 70% ethanol. The remaining

wells in the plates were then examined for cell growth using an inverted microscope (Diavert, Leitz, Wetzlar). Wells which showed definite focal colonies growth were marked for subsequent examination. At the same time partial medium supplement was made by adding 50 μl fresh warmed HAT medium to each well in the plates.

On day 10 after the fusion, the plates were again screened for cell growth and any overlapped hybrids were released by carefully sucking them up and down with a pipette. Next day, hybridoma culture supernatants were harvested from wells marked as showing good cell growth on day 10 by removing 100 μl from each marked well and replacing it with 100 μl fresh HAT medium. Parameters used to indicate that the time was appropriate for antibody screening included cell growth covering at least 70% of the microtitre well bottom and medium colour change towards yellow.

Harvested undiluted supernatants were subjected to an initial IFAT screen against formalin fixed *T. evansi* trypanosomes utilising FITC-labelled sheep anti-mouse IgG (The Binding Site Ltd, Birmingham, U.K) as a conjugate as described in section 2.10.3.

Expansion of hybridoma cultures:

Cultures from which the supernatants gave positive reaction by IFAT in the initial screen were expanded into 2 cm^2 wells (24 well plates, Gibco). For this the microtitre well contents were resuspended and transferred to a 2 cm^2 well containing 1 ml HAT medium. The original microtitre well culture continued to be maintained by regular addition of fresh medium until the cell cultures derived from it were established in the larger vessels and cryopreserved. Culture supernatants from hybridoma cells growing in the 2

cm^2 wells, were tested for antibody activity against *T. evansi* by IFAT. Cells from positively reacting wells were then cloned, cryopreserved and expanded further. This expansion involved the transfer of the 2 cm^2 well containing cloned cells to 25 cm^2 tissue culture flasks and thence to 75 and 150 cm^2 flasks. At all stages cultures in the original wells were maintained and culture supernatants were tested for antibody activity by IFAT at regular intervals.

Cloning of hybridoma cultures:

Cloning was achieved by limiting dilution (Oi and Herzenberg, 1980) in 96 well microtitre plates. The cultures from 2 cm^2 wells from 3 cell lines were selected for cloning based on their reaction in IFAT. Cells were resuspended and the cell concentration and viability determined using trypan blue and haemocytometer counting of cells. Dilutions of the cells were then prepared to a potential 5 cells per ml in HAT medium. The dilutions were then plated out as follows as 100 µl/well, in plates previously coated with feeder cell layer at a concentration of $5x10^6$ cells/ml:

20 wells @ 50 cells/ml i.e. 5 cells/well.

20 wells @ 10 cells/ml i.e. 1 cell/well.

20 wells @ 5 cells/ml i.e. 0.5 cells/well.

Seven days after cloning, microtitre plates were screened for growth using an inverted microscope. Wells containing cloned colonies deriving from a single cell were marked and the cells were fed by addition of 100 µl HAT medium. When the cells were near to confluence the culture supernatants were removed and tested for antibody activity by IFAT against formalin-fixed *T. evansi* trypanosomes. Antibody-positive cloned cultures were maintained in

HAT medium until transfer to 2 cm^2 wells. Thereafter, the medium was gradually changed to HT medium. Passages were carried out every 2-3 days and the HT medium was gradually changed to complete medium and the cloned cultures were finally grown in complete medium. The cloning was performed at least twice on the same culture using the above procedure.

Cryopreservation of hybridoma cultures:

Cells from confluent cultures were centrifuged for 5 minutes at 1000 r.p.m. The pelleted cells were then suspended in ice-cold freezing medium (10% dimethyl sulphoxide in foetal calf serum) and cryopreserved in 1 ml aliquots in cryotubes (Gibco) pre-cooled at -20°C. The tubes were placed in a foam-insulated box and stored for 24 hours at -70°C and then transferred to a liquid nitrogen bank for long term storage.

Resuscitation of hybridoma cultures:

Cryopreserved hybridoma cells were thawed in a 37°C water bath and immediately after thawing a few drops of complete medium were added to the cell suspension over a period of 1 - 2 minutes. The contents were then made up to 10 ml with complete medium, mixed and transferred to 25 cm^2 tissue culture flask and incubated in a humidified 37°C incubator in atmosphere of 5% CO_2.

Harvesting monoclonal antibodies:

McAbs were produced in hybridoma culture supernatants or in ascites fluids obtained from hybridomas grown in syngeneic Balb/C mice. Culture supernatants were collected from the 75 or 150 cm^2 flasks in 15-25 ml volumes during passage after either 48 or 72 hours cell growth. The culture supernatant was centrifuged at 2500 r.p.m. at 4°C and then stored at -20°C.

For ascites production, mice were given i.p. injections of 0.5 ml pristane (2,6,10,14-tetramethylpentadecane, Sigma) one week before inoculation i.p. with $5x10^6$ live hybridoma cells in complete medium obtained from cultures in the exponential phase of growth. The hybridoma was allowed to grow in the mice for 1 - 4 weeks. A puncture was made in the peritoneal cavity of the mice using a 21-gauge needle and drops of the ascitic fluid were collected in small tubes. 2-3 ml of ascitic fluid were collected per mouse and was centrifuged at 3000 r.p.m. for 10 minutes at 4°C and stored at - 20°C.

Nomenclature of hybridoma:

Parent cell lines were named after the original fusion plate and wells from which they were selected, e.g. 2D5. Clones names include parent line and the well of the limiting dilution plates from which the clone was taken e.g 2D5/E2/C7.

Antibody characterisation:

Antibody-ELISA:

ELISA was used to test the reactivity of the McAbs against the soluble *T. evansi* antigens and to determine the antibody titre in the serum collected before fusion. The microtitre plates were coated overnight with a soluble extract of the homologous *T. evansi* population (TREU 2165) at a dilution of 1/80 and processed as in section 2.8. The ascites and hybridoma culture supernatants containing the McAbs at a 2-fold dilution range from 1/10 to 1/320 or the test and normal mouse serum diluted from 1/640 to 1/20480 were then added to the plates, incubated and washed as described in section 2.8. A peroxidase-labelled goat anti-mouse IgG (Pierce, USA) diluted to 1/5000 in PBS/Tween was then added, incubated and washed as above before the TMB substrate solution was added and incubated for 15 minutes at 37°C. The

substrate reaction was stopped with 2M sulphuric acid and the optical density of the wells read at 450 nm using an ELISA plate reading photometer.

Immunoblotting:

Western immunoblotting was used to determine the molecular nature of the antigen recognised by the McAbs in the whole trypanosome antigen preparation of the homologous *T. evansi* population (TREU 2165). One McAb, 2D5/E2/C6/C7, was also tested against two heterologous populations of *T. evansi* (TREU 2222 and TREU 2257). Soluble extracts of the trypanosomes were fractionated by SDS-PAGE (section 2.7) and then electrophoretically transferred to a nitrocellulose membrane (section 2.7.1.). The proteins bound to the nitrocellulose membrane were probed with ascites, for each McAb, at a dilution of 1/20 or hybridoma culture supernatant diluted to 1/2. The pre-fusion serum from the mouse at a dilution of 1/20 was also included in the assay as a positive control. The procedure of western immunoblotting was performed as described in section 2.7.1.

Agglutination Test:

The agglutinating ability of the McAbs was investigated against the homologous population, TREU 2165 and two heterologous populations TREU 2222 and TREU 2257 of *T. evansi*. The ascites and culture supernatants were tested over a 2-fold dilution range ranging from 1/2 to 1/1024. Details of the test are described in section 2.9.

5. 4. 2. RESULTS:

The pre-fusion serum from the mouse that supplied the spleen had high levels of IgG antibodies with an ELISA titre of 1/20000 when tested against the homologous antigen.

Primary screen of the hybridoma cultures and production of McAbs

Seven days after the first fusion the hybridoma cultures in all plates were found to be contaminated and were discarded. A second fusion was performed 13 days later.

Ten days after the second fusion, 268 of the 360 microtitre wells that received fused cells contained distinct colonies. Forty five of these colonies were positive for anti-*T. evansi* antibody when the culture supernatants were tested by IFAT using formalin-fixed *T. evansi* (TREU 2165) trypanosomes. Subsequently the 45 antibody-secreting hybridomas were expanded and cryopreserved. Three lines were produced from these hybridomas and cloned. Finally 4 stable cloned hybridoma cell lines (2D5/B10/C7, 2D5/B10/E4, 2D5/E2/C6 and 2D5/E2/C7) were established as secreting antibodies to the homologous *T. evansi* population by IFAT.

Characterisation results:

Culture supernatant from 4 clones showed an absorbance value similar to that of PBS against the homologous population of *T. evansi* in antibody-ELISA. The ascitic fluid produced from these cells reacted with the homologous population of *T. evansi* in ELISA (Table 5.3). The McAb 2D5/E2/C7 showed the strongest reaction as defined by the absorbance of the antigen-antibody reaction followed by 2D5/B10/C7, 2D5/B10/E4 and 2D5/E2/C6 (Table 5. 3)

The ascites of each of the four McAbs recognised a single antigenic component of molecular weight of 72 k.Da in the homologous population of *T. evansi* (TREU 2165) when tested by immunoblotting (Figure 5.1 and Table 5.3). The McAb 2D5/E2/C7 showed the strongest reaction with this

component as determined by the staining intensity of the protein band (Figure 5.1 Lane 7), while the other 3 antibodies showed a reaction of low staining intensity. The hybridoma culture supernatant of the 4 McAbs did not show any reaction by immunoblotting (Figure 5.1). The McAb 2D5/E2/C7 recognised the same antigenic component in a heterologous variant of *T. evansi* (TREU 2222) but not in the heterologous stock TREU 2257 (Figure 5. 2).

A low agglutination of titre of 1/16 was obtained only with the homologous *T. evansi* population (TREU 2165) when the ascites containing the McAb 2D5/E2/C7 was analysed by the agglutination test. No agglutination was detected when either of the other three monoclonals were analysed by this method (Table 5. 3). The heterologous populations of the parasites were not agglutinated by any of the 4 antibodies.

Table 5. 3

Reaction of the four McAbs with the homologous *T. evansi* population

Hybridoma number	Reactivity in ELISA		Immunoblotting		Agglutination	
	A	S	A	S	A	S
2D5/E2/C7:	0.710	0.071	72 k.Da	-	1/16	-
2D5/B10/C7:	0.681	0.066	72 k.Da	-	-	-
2D5/B10/E4:	0.462	0.068	72 k.Da	-	-	-
2D5/E2/C6:	0.320	0.067	72 k.Da	-	-	-

A ≡ Ascites.

S ≡ Supernatant.

- ≡ Negative reaction.

Figure 5. 1

Reaction of the four McAbs with the homologous population of *T. evansi* in immunoblotting

Lane 1: 2D5/B10/E4 ascitic fluid.

Lane 2: 2D5/B10/E4 culture supernatant.

Lane 3: 2D5/B10/C7 ascitic fluid.

Lane 4: 2D5/B10/C7 culture supernatant.

Lane 5: 2D5/E2/C6 ascitic fluid.

Lane 6: 2D5/E2/C6 culture supernatant.

Lane 7: 2D5/E2/C7 ascitic fluid.

Lane 8: 2D5/E2/C7 culture supernatant.

Lane 9: Pre-fusion serum.

Lane 10: Molecular weight markers.

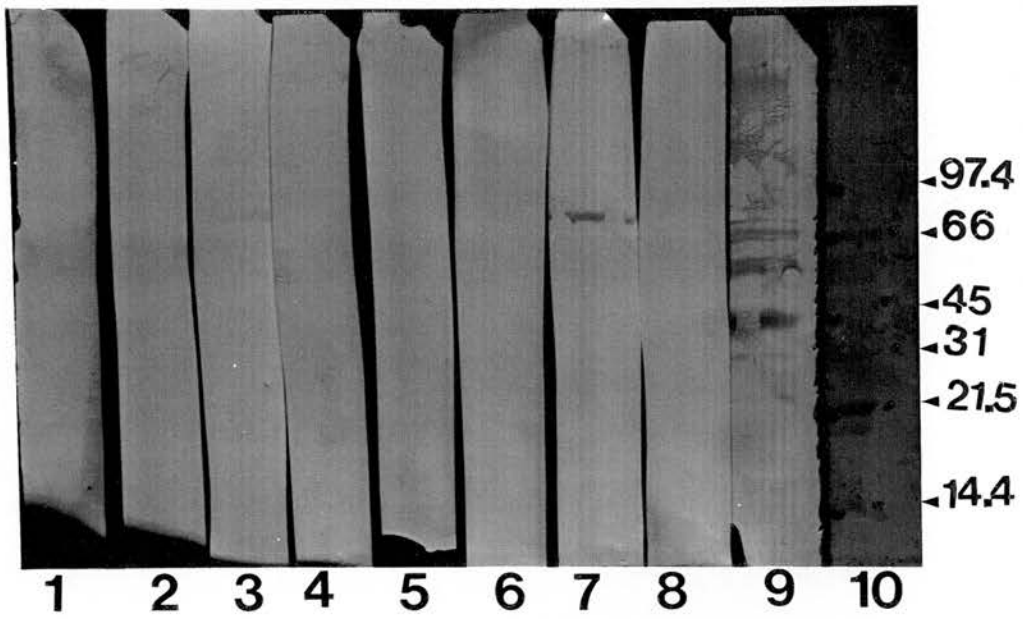

97.4
66
45
31
21.5
14.4
1
2
3
4
5
6
7
8
9
10

Figure 5. 2

McAb 2D5/E2/C7: Antibody specificity probed by immunoblotting against the homologous population of *T. evansi* (TREU 2165) and the heterologous populations (TREU 2222 and TREU 2257) fractionated by SDS-PAGE on a 10% acrylamide.

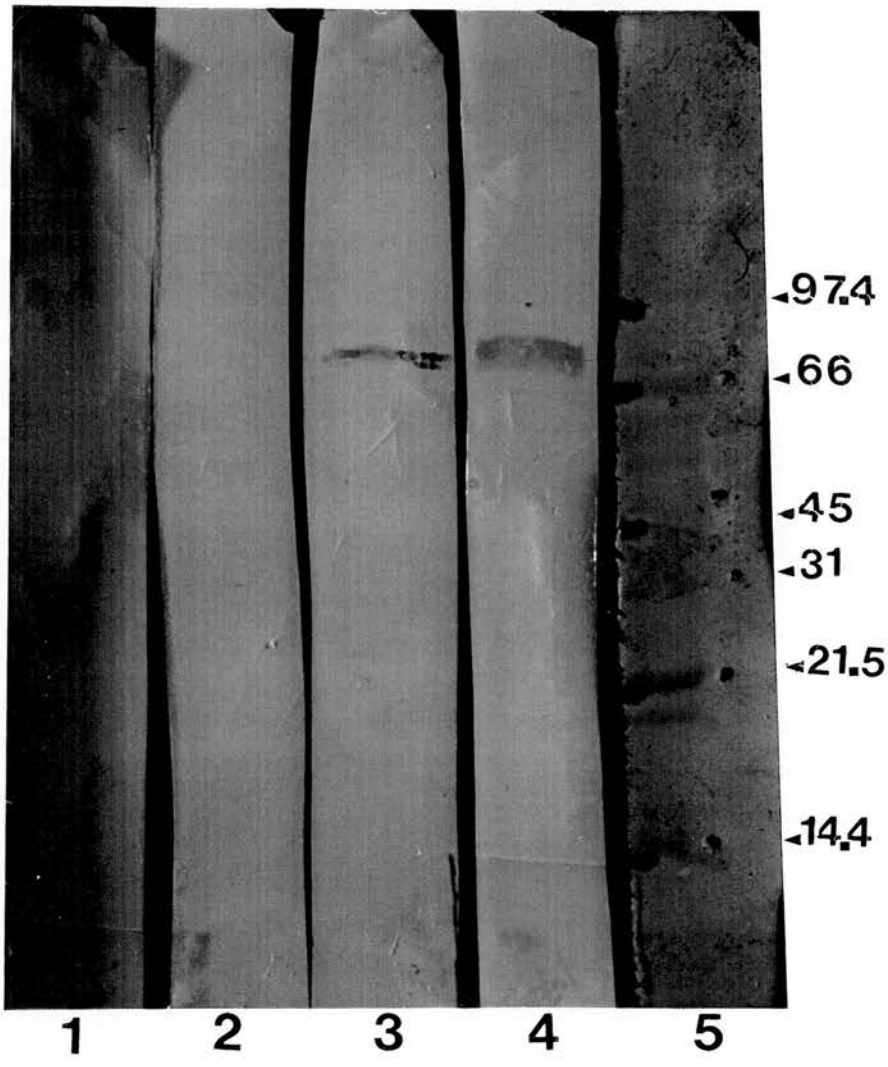
Lane 1: Normal mouse serum.

Lane 2: TREU 2257 (heterologous stock).

Lane 3: TREU 2222 (heterologous variant).

Lane 4: TREU 2265 (homologous population).

Lane 5: Molecular weight markers.


97.4
66
45
31
21.5
14.4
1
2
3
4
5

5. 4. 3. DISCUSSION:

The immunisation regime used for the Balb/C mice in the present study elicited a high level (ELISA titre of 1/20000) of IgG antibodies to *T. evansi* in the serum collected before fusion. Immunisation protocols employing infection, treatment and boosting with living trypanosomes have previously been successfully used to produce McAbs to variant and invariant trypanosome antigens (Masterson et al, 1988, Burgess and Jerrells, 1985). The immunisation regime used for the production of McAbs to *T. evansi* in the present study was slightly longer than that used in the pilot study as the first fusion performed was contaminated and a second fusion had to be undertaken.

The second fusion was successful and a high proportion of fused cells secreting antibodies to *T. evansi* was produced with a minimum of contamination. Four anti-*T. evansi* monoclonal antibodies were finally established from this fusion. This low number was obtained despite the fact that 3 parent cultures were cloned and is a reflection of the technical difficulties in handling many secreting lines. Many cultures previously identified as anti-*T. evansi* antibody secretors either stopped secreting antibodies or ceased to grow before they could be preserved. Other workers have reported similar numbers of 2-5 McAbs per fusion from trypanosomes (Masterson et al, 1988, Turner et al, 1989). The loss of antibody secreting ability prior to cloning is a well established phenomenon often resulting from overgrowth of secretors by non-secreting hybridomas especially as antibody production, by its use of cell resources, makes a secreting hybridoma cell line less able to compete for growth with non-secretors (Goding, 1980). Early cloning as practised in the past can prevent this, but cessation of secretion is still a frequent occurrence as in present study. Loss of chromosomal materials

(Goding, 1980) or instability in gene expression in hybridoma cells (Pearson *et al*, 1980; Galfre and Milstein, 1981) attributed to loss of antibody secreting ability in a cloned cell line.

The four McAbs produced recognised a similar antigenic component of 72 k.Da by immunoblotting in the homologous population of *T. evansi*. This antigenic component was also recognised in a heterologous variant of the same *T. evansi* stock indicating that these McAbs recognise stock-specific antigen. In the IFAT used to screen the hybridomas an overall green fluorescence was observed on the formalin-fixed trypanosomes suggesting that the antigen recognised by these McAbs may be located on the surface of the parasite since formalin-fixation is reputed to stabilise the surface antigens (Nantulya and Doyle, 1977). It could, however, also be of a non-surface origin since internal antigens would also be exposed following fixation and drying of the trypanosomes and in this case the antigen is dispensed in the cytoplasm and not localised in organelles during IFAT screening. The non-agglutinating ability of these McAbs observed in this study also suggest that this 72 k.Da antigen is not exposed on the surface of the parasite or it is present in small amount that could not induce agglutinating antibodies. A 72 k.Da antigen was also recognised by the 21 days serum raised in rabbits against the homologous population of *T. evansi* in earlier part of the study (see section 3) and by the pre-fusion serum in the present study (Figure 5. 1) indicating that this component is recognised by the infected hosts as an antigen during the course of infection. The 72 k.Da antigen described in section three suggest that it is a soluble antigen which is insensitive to trypsin again indicating that this antigen is possibly not located on the surface of the parasite or do not posses arginine and lysine residues that are sensitive to trypsin digestion.

The hybridoma culture supernatants collected from the cloned cultures did not react in either ELISA or immunoblotting possibly due to the low concentration of antibodies in these supernatants as the cells were grown in large volumes of medium.

The present study produced McAbs to a stock-specific 72 k.Da antigen of *T. evansi* that is antigenic during the course of infection, hence these antibodies are valuable reagents for the diagnosis of infection and epidemiological studies of this stock of *T. evansi*. The antibody produced will be used to develop immunoassays for the detection of the 72 k.Da antigen in serum and tissues of infected animals.

CHAPTER SIX

PRODUCTION OF LABELLED ANTIBODIES TO *T. EVANSI*

6. 1. INTRODUCTION:

The efficiency of an immunoassays is directly related to the purity of the antibodies used (Tijssen, 1985). Serum, ascitic fluid and hybridoma culture supernatants can contain a wide range of proteinaceous substances in addition to immunoglobulins. and are not usually suitable for direct use in immunoassays for antigen detection. In practice, therefore, the antigen-specific immunoglobulins must be purified from these materials. This is particularly important if the antibodies have to be labelled because it reduces the amount of the label required by as much as 10-fold (Tijssen, 1985).

IgG is the immunoglobulin class most commonly used in immunoassays as most IgG antibodies are of higher affinity to the immunogen than IgM (Harlow and Lane, 1988). High-affinity antibodies bind large amounts of antigen and consequently produce a strong signal in a short period of time in immunoassays (Harlow and Lane, 1988). Low affinity-antibodies bind only a small proportion of the antigen and therefore require a longer period of time to develop a detectable signal. The affinity of the antibody also has an effect on the avidity of the antigen-antibody complex because antibodies with a high affinity increase the stability of the antigen-antibody complex during washings (Tijssen, 1985), therefore increasing the final signal strength.

IgG is much easier to purify compared to IgM, IgA, IgD and IgE (Goding, 1986). A number of methods have been developed for the purification of IgG usually governed by the intended application of the purified antibody, the animal species in which the antibody was raised and whether the starting material is serum, ascites or tissue culture supernatant (Harlow and Lane, 1988). Simple methods such as ammonium sulphate

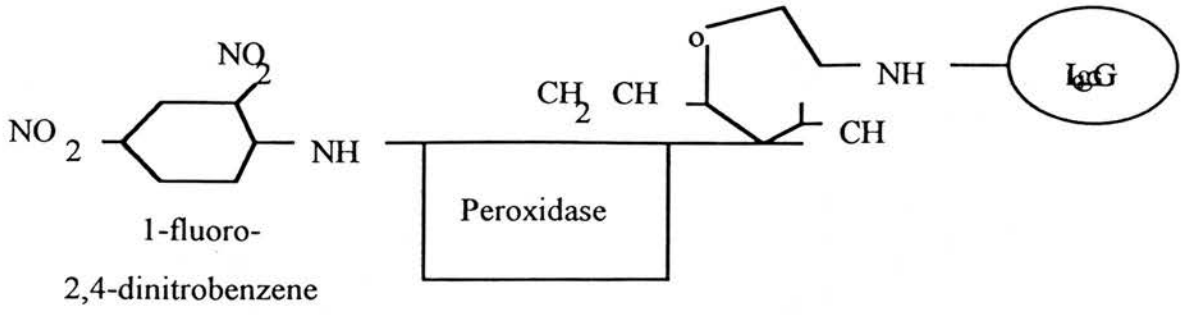
precipitation and DEAE chromatography are capable of separating antibodies from a wide range of sources but do not yield pure IgG (Harlow and Lane, 1988). Affinity chromatography using IgG-specific ligands such as protein A or G coupled to sepharose beads can produce pure IgG in high yield (Tijssen, 1985, Harlow and Lane, 1988). A disadvantage of this method, however, is that IgG from different species and individual IgG subclasses differ in their affinity to these ligands (Tijssen, 1985), with subsequent differences in yield and purity of the IgG. To some extent this problem can be solved by increasing the salt concentration of the binding buffer which increases the affinity of these IgG for protein A (Harlow and Lane, 1988). This phenomemon has been exploited in commercially available kits for IgG preparation such as those produced by the Pierce Chemical Company who have developed a custom system of buffers. These are claimed to enhance the binding of IgG to immobilised protein A from a wide range of sources including ascitices fluid and serum from different species.

After purification from contaminating proteins, the antibodies for use in immunoassays usually require to be labelled with an easily detected tag so that any antigen-antibody complex can be detected. The label for such complexing can be placed either directly onto the antibody or indirectly by first coupling the antibody to small chemical group such as biotin which, in turn, can be detected by a labelled ligand such as avidin. Different types of labelling systems are available depending on the proposed assay. Fluorochromes, such as fluorescein isothiocyanate (FITC) and tetramethyl rhodamine isothiocyanate (TRITC) are generally suitable for immunohistochemistry and flow cytometry (Johnstone and Thorpe, 1982). Radioisotopes such as iodine (^{125}I) are used in radioimmunoassays (Liddell and Cryer, 1991), while enzymes are used for increasingly popular enzyme-immunoassays.

Conjugation of the antibody to the label without impairing the activity of either molecule is vital. The active site of most enzymes usually occupies a relatively small part of the enzyme which means that the remaining portion is available for conjugation to the antibody without affecting the catalytic activity of the enzyme. Enzymes have achieved a high degree of popularity in immunoassays mainly because of their high specificity for the substrate which can be converted to coloured products that can be either soluble or insoluble. Soluble products are needed for use in ELISA-type assays and insoluble products are for immunoblotting and immunohistochemistry. Enzymes also have the advantage over other labels in that they are readily available, inexpensive and have high specific activity (Tijssen, 1985). The most commonly used enzymes include horseradish peroxidase (HRP), alkaline phosphatase (AP) and β-galactosidase (β-G) (Harlow and Lane, 1988). Overall, HRP conjugates are considered to be superior to AP and β-G conjugates due to the higher specific enzyme activity and immunological reactivity of HRP conjugates (Porstmann *et al*, 1985). The reaction products of HRP are easily detectable, while that of AP and β-G are less strongly coloured (Liddell and Cryer, 1991).

There are a number of methods used to couple HRP to immunoglobulin, however, the periodate method (Wilson and Nakane, 1978) is the most commonly used. This method is popular because it couples the antibody to HRP through the carbohydrate portion of each molecule as shown in the diagram below and, since this is not usually involved in the active site of the antibody or the enzyme, the method is less likely to reduce the immunoreactivity.

Peroxidase-IgG conjugate prepared by the periodate method



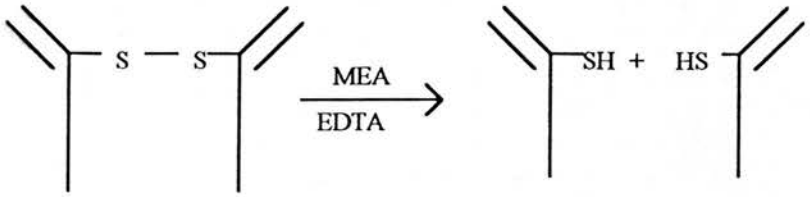
Another popular method of conjugating antibody to HRP is the two-step gluteraldehyde method developed by Avrameas and Ternynck (1971). This method links the amino groups of lysine residues [Antibody - N = C′-$(CH_2)_3$ - C′ = N - enzyme] and although simple to perform is considered inferior to the periodate method in terms of yield (Voller *et al*, 1979) and titre of conjugates (Nygren, 1982).

The indirect method of labelling depends on the antibodies being modified by the addition a small molecule such as biotin. This molecule can then be detected by either of the biotin-binding proteins, avidin or streptavidin, which have themselves been labelled with HRP, AP or β-G (Harlow and Lane, 1988). In this way a larger number of labelled molecules can be attached to the antibody than is possible by direct labelling which greatly improves the efficiency of the assay (Tijssen, 1985). Avidin has a high affinity for biotin and the bond formation between them is very stable (Tijssen, 1985), and hence will not be released during immunoassay washing cycles. A number of biotin derivatives are commercially available for biotinylating antibodies. In this study Biotin-HPDP derivative a sulfhydryl specific reagent was used because it preserve the antigen binding site of the immunoglobulin since it couples

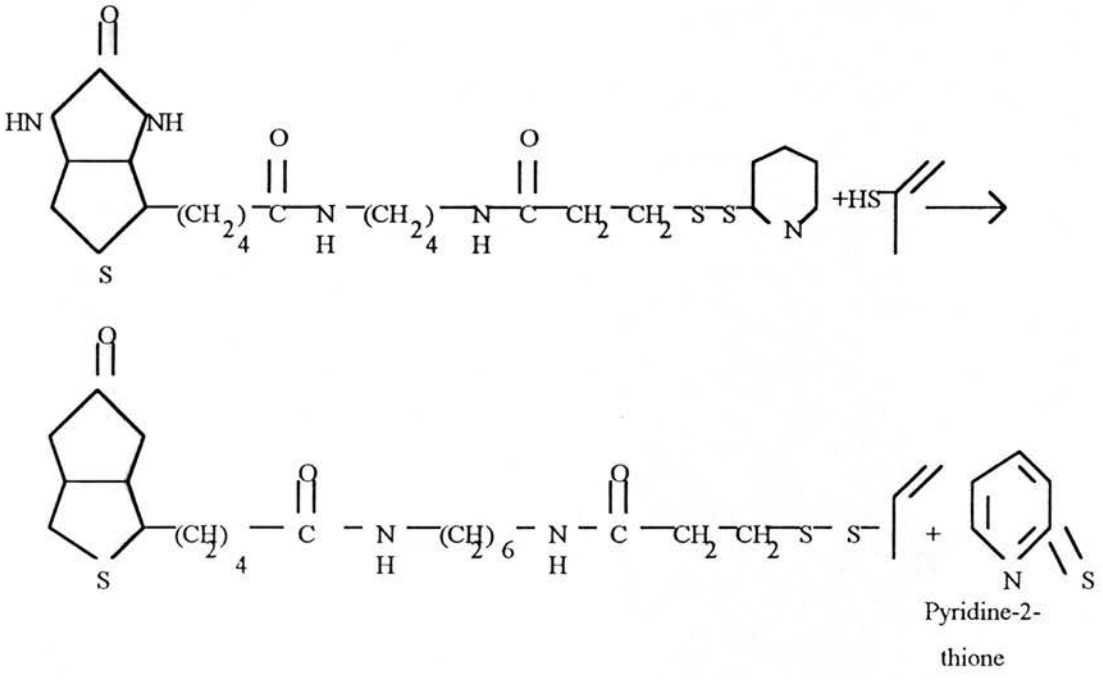
through the sulfhydryls at the hinge region of the reduced immunoglobulin as shown in the diagrams below.

Biotinylation of IgG

(1) Reduction of antibody using mercaptoethylamine (MEA):



(2) Addition of biotin-HPDP to the reduced antibody results in the linkage of biotin to the antibody via the sulfhydryl groups in the hinge region.



The present study investigated the affinity chromatographic purification of IgG molecules from rabbit serum and ascitic fluid using Protein A chromatography along with methods for the labelling of the purified IgG with HRP or biotin.

6. 2. MATERIALS AND METHODS:

6. 2. 1. Purification of Antibodies from Serum and Ascites fluid:

The antibodies from the serum raised in rabbits against the *T. evansi* 42 k.Da and 52 k.Da antigens (section 4.2.6) and the ascitic fluid containing the 72 k.Da monoclonal antibody 2D5/E2/C7 were purified using the commercially available ImmunoPure^{R} IgG kit (Pierce Chemical Company, USA). The method utilised 1.0 ml columns of immobilised protein A covalently coupled to 6%, crosslinked, beaded agarose (Protein A affinityPak ™ columns).

In this method 2.5 ml of the serum sample or ascites fluid were diluted with 2.5 ml of ImmunoPure^{R} IgG binding buffer, pH 8.0. The Protein A affinityPak™ column was equilibrated with 5 ml of ImmunoPure^{R} IgG binding buffer before the diluted sample was applied to the column. The sample was allowed to flow completely into the column before washing it with 15 ml of ImmunoPure^{R} IgG binding buffer. The IgG bound to the column was eluted in 5x1 ml fractions of ImmunoPure^{R} IgG elution buffer, pH 2.8. The elution was monitored by measuring the absorbance of each fraction at 280 nm. Fractions eluted from the Protein A affinityPak™ column that showed absorbance values greater than 0.5 at 280 nm were each dialysed against PBS and then stored at -20^{o}C. Three fractions were collected from the anti-42 k.Da and anti-52 k.Da serum and a single fraction from the monoclonal antibody.

Antibody reactivity:

The dialysed fractions from the protein A affinityPak™ columns were each tested against freeze-thawed antigens from the homologous *T. evansi* population by ELISA. The antigen was used at a dilution of 1/80. The anti-42

and 52 k.Da fractions were tested at six 2-fold dilutions from 1/400 to 1/12800. The anti-72 k.Da fractions were tested at six 2-fold dilutions 1/10 to 1/320. The antigen-antibody complex was detected by peroxidase-labelled donkey anti-rabbit IgG or goat anti-mouse IgG at a dilution of 1/5000 and the reaction was visualised by TMB substrate as described in section 2.8.

6. 2. 2. Labelling of antibodies:

For all three antibodies, the second fraction eluted from the Protein A affinityPak™ column had both the highest absorbance value at 280 nm and greatest antibody reactivity when tested against the freeze-thawed *T. evansi* antigens by ELISA. These fractions were, therefore, chosen for labelling studies. Before labelling the selected IgG fractions were concentrated by freeze-drying. Prior to this each fraction was dialysed with slow stirring overnight against several changes of distilled water at 4°C. The dialysed fractions were frozen onto the sides of 5 ml bijoux in a bath of methanol cooled with solid CO_2 and then freeze-dried overnight using a Virtis freeze-dryer (Medicell International Ltd.).

Labelling of IgG with horseradish peroxidase (HRP):
The modified periodate method:

The modified periodate method (Wilson and Nakane, 1978) was used to conjugate HRP to antibodies to the three antigens. In this method, 50 µl of a 38.5 mg/ml solution of sodium periodate ($NaIO_4$) was added to 4 mg of HRP (Type VI, activity 250-330 units/mg, Sigma Ltd. UK) dissolved in 1 ml distilled water. The mixture was stirred for 20 minutes at room temperature to oxidise the carbohydrate residues of the HRP and then dialysed at 4°C overnight against 1 mM sodium acetate buffer, pH 4.4. The activated HRP was transferred to a 10x75 mm test tube, and 20 µl of 0.2M sodium carbonate

buffer, pH 9.5 was added followed immediately by 10 mg of the relevant lyophilised antibody fraction dissolved in 0.01M sodium carbonate buffer, pH 9.5. The reaction mixture was stirred for 2 hours at room temperature and then 40 mg of lysine was added to block any remaining unreacted aldehyde groups. 100 µl of freshly prepared sodium borohydride ($NaBH_4$, 4 mg /ml distilled water) was then added and the mixture incubated for a further 2 hours at 4°C. The resulting IgG-HRP conjugate was dialysed overnight against PBS at 4°C. Prior to storage an equal volume of glycerol was added to the conjugate which was then stored at -20°C until needed.

ImmunoPure^R activated peroxidase kit

The ImmunoPure^R activated peroxidase kit (Pierce, USA) was used to conjugate the anti-42 k.Da immunoglobulins to HRP. In this method 0.3 mg lyophilised antibody was dissolved in 100 µl of coupling buffer (0.1 M $NaHCO_3$, 0.9% NaCl, pH 9.5) and added to 1 mg of the HRP previously dissolved in 100 µl distilled water. After incubation overnight at 4°C, the reaction was stopped by the addition of 40 mg lysine followed by a 2 hour incubation at room temperature. The reaction mixture was then mixed with an equal volume of immunoPure^R gentle binding buffer, pH 8.0 (Pierce, USA) and applied to a column containing a mixture of immobilised protein A and protein G which had been pre-equilibrated with 5 ml of the immunoPure^R gentle binding buffer. The column was then washed with immunoPure^R gentle binding buffer and 15 x 1 ml fractions collected. The absorbance of the column fractions was monitored at 280 nm to ensure that a stable base line was reached. The reaction of these 15 x 1 ml fractions to the substrate TMB was also monitored by adding a 100 µl aliquot of each fraction to 100 µl of the TMB substrate (section 2.8). The HRP-conjugated IgG bound to the column was then eluted with immunoPure^R gentle elution buffer; 10 x 1 ml fractions

were collected and the elution of the conjugate from the column was monitored at 280 nm. The conjugate fractions were then dialysed against 50 mM Tris buffer, pH 7.5 containing 0.9% NaCl, 0.2% thimerosal; 10 mg of BSA was added to the dialysed conjugate as a stabiliser.

Conjugate immunoreactivity test:

Antibodies conjugated to HRP through the modified periodate method and the 10 fractions of the anti-42 k.Da antibodies conjugated by the Pierce immunoPure^{R} activated peroxidase kit were tested against the freeze-thawed antigens of the homologous *T. evansi* population at a dilution of 1/80 using direct ELISA. The conjugated antibodies were tested at 2-fold dilutions (table 6.1) and the reaction was visualised using TMB substrate as described in section 2.8.

Table 6. 1

Dilutions of the HRP-labelled antibodies used in the immunoreactivity test

	Conjugates dilutions		
Labelling method	42 k.Da	52 k.Da	72 k.Da
Modified periodate method	1/500-1/6400	1/50-1/3200	1/10-1/320
Activated peroxidase kit	1/10-1/320		

Labelling IgG with biotin:

The 42 k.Da and 52 k.Da IgG molecules were coupled to biotin using N-[6-(Biotinamido)hexyl]-3′-(2′-pyridyldithio) proionamide(biotin-HPDP derivative Pierce Chemical Company, USA). In this method 10 mg lyophilised IgG was dissolved in 0.5 ml of 0.1M sodium phosphate buffer, pH 6.0 containing 5 mM EDTA. The IgG was then reduced by mixing with 3.5 mg of 2-mercaptoethylamine HCl and incubating for 90 minutes at 37°C. The solution was cooled to room temperature and excess mercaptoethylamine was removed by a desalting column. The reaction mixture was applied to a 5 ml desalting column (Excellulose prepacked GF-5 desalting column, Pierce, USA) that had been pre-equilibrated with PBS-EDTA buffer, pH 7.4 (20 mM sodium phosphate, 150 mM NaCl, 1 mM EDTA). The column eluate was discarded. The column was then washed with 0.5 ml PBS-EDTA. A 1.25 ml volume of PBS-EDTA was then added to the column and the eluate, which contained the reduced IgG was collected in a single fraction.

A 4 mM biotin-HPDP stock solution was prepared by dissolving 2.2 mg biotin-HPDP in 1 ml dimethylformamide. To 50 µl of this stock solution 1 ml (8 mg) of the reduced IgG was added, the mixture vortexed and then incubated for 90 minutes at room temperature. The progress of the reaction was monitored by following the change in absorbance at 343 nm for 15 minutes due to the release of Pyridine-2-thione as a result of the reaction between biotin and the reduced IgG. The resulting biotinylated IgG was then purified by applying the mixture to a GF-5 desalting column as described above. The column was washed with 1 ml PBS-EDTA; a 2.5 ml volume of PBS-EDTA buffer was then added and the eluate, which contained the biotinylated IgG was collected in a single fraction and stored at - 20°C.

Biotinylated antibodies immunoreactivity test:

The biotinylated anti-42 and 52 k.Da IgG were tested against the freeze-thawed antigen of the homologous *T. evansi* population at a dilution of 1/80 using a direct ELISA. The biotinylated IgGs were tested at eight 2-fold dilutions from 1/10 to 1/1280. The antigen-biotinylated IgG complex was detected using avidin coupled to HRP (Pierce, USA) at a dilution of 1/10000. The reaction was visualised using TMB substrate as described in section 2.8.

6. 3. RESULTS:

6. 3. 1. Purification of Antibodies:

The 280 nm absorbance values of the fractions collected from the protein A affinityPak™ columns are shown in table 6. 2. For the anti-42 k.Da antibodies the major antibody peak in the column elution occured in the second eluate fraction with an absorbance value of 2.472 at 280 nm. The first and the third fractions showed lower absorbance values and the elution of antibody was considered to be completed by the third fraction. A similar elution pattern was seen with the anti-52 k.Da antibodies and the anti- 72 k.Da antibodies with major antibody peaks seen in fraction 2 with an absorbance of 2.458 and 2.180 at 280 nm respectivly.

The first three fractions from the anti 42 and 52 K.Da serum and the second fraction from the anti-72k.Da ascitic fluid were used for further studies on labelling efficiencies.

Table 6. 2

Purification of *T. evansi* antibodies using protein A affinityPak™ columns (Pierce Chemical Company, USA)

	Absorbance at 280 nm		
	anti-42 k.Da	anti-52 k.Da	anti-72 k.Da
Fraction 1:	0.656	0.965	0.010
Fraction 2:	2.472	2.458	2.180
Fraction 3:	1.723	1.204	0.097
Fraction 4:	0.086	0.034	0.000
Fraction 5:	0.000	0.000	0.000

Antibody reactivity:

The ELISA absorbance values of the three fractions of the anti-42 k.Da antibody decreased with increasing dilutions of the antibody when titrated against soluble extract of the homologous *T.evansi* population. The highest activity was seen in the second eluate fraction with an absorbance of more than 1.0 at a dilution of 1/1600 (Table 6.3). The first and the third fractions

showed much lower antibody activity with an absorbance values of 0.149 and 0.132 respectivly at a dilution of 1/1600 (Table 6.3).

Similarly, the greatest antibody activity of the anti-52 k.Da preparation was seen in the second fraction (Table 6.3) with an absorbance value of more than 1.0 but only at a dilution of 1/400. Again the first and third fractions showed much lower absorbance values (0.241 and 0.302 respectively) at this dilution.

As in the case of the other two preparation the second fraction collected from the anti-72 k.Da antibody showed highest activity against the homologous *T. evansi* population with an absorbance value of 0.962 at a dilution of 1/10 (Table 6.3).

Consequently the second fraction from each antibody preparation was selected for further study.

Table 6. 3: *T. evansi* antibodies purified by protein A affinityPak™ columns (Pierce, USA): Reaction of eluate fractions with the homologous *T. evansi* population in ELISA.

	Antibody dilutions					
	1/400	1/800	1/1600	1/3200	1/6400	1/12800
Anti-42 k.Da:						
Fraction 1	0.315	0.201	0.149	0.127	0.101	0.097
Fraction 2	1.898	1.379	1.001	0.645	0.355	0.239
Fraction 3	0.242	0.165	0.132	0.129	0.102	0.120
Anti-52 k.Da:						
Fraction 1	0.241	0.167	0.115	0.079	0.067	0.069
Fraction 2	1.199	0.994	0.660	0.422	0.247	0.172
Fraction 3	0.302	0.217	0.131	0.092	0.071	0.062
Anti-72 k.Da dilution:	1/10	1/20	1/40	1/80	1/160	1/320
Fraction 2	0.962	0.596	0.445	0.264	0.198	0.182

6. 3. 2. Antibody-labelling:

HRP labelling by the modified periodate method:

HRP conjugates produced by the modified periodate method reacted with the homologous *T. evansi* antigens when tested by a direct ELISA which titrated out with increasing conjugate dilution (Figure 6. 1). The absorbance values obtained with the anti-42 k.Da conjugate ranged from 1.803 at a dilution of 1/500 to 0.079 at 1/64000 (Figure 6.1A) while values with the anti-52 k.Da preparation ranged from 1.713 at a dilution of 1/50 to 0.105 at a dilution of 1/3200 (Figure 6.1B). Absorbance values with the anti-72 k.Da conjugate ranged from 1.981 to 0.288 but over a lesser dilution range of 1/10 to 1/320 (Figure 6.1C).

The anti-42 k.Da conjugate showed an estimated titre of 1/2000 at an absorbance value of 1.0 while the anti-52 k.Da conjugate had an estimated titre of 1/200 for similar absorbance value (Table 6.4). The anti-72 k.Da McAb conjugate had an estimated titre of 1/80 at an absorbance value of 1.0 (Table 6.4). The molecular concentration ratio of IgG:HRP used for each conjugate was 2:3.

Table 6.4

Comparison of the activity of the anti-*T. evansi* HRP-labelled antibodies

Antigen	Starting dilution	Absorbance at starting dil.	Dilution at an absorb. of 1.0	Antibody: enzyme ratio
42 k.Da	1/500	1.803	1000-2000	2:3
52 k.Da	1/50	1.713	100-200	2:3
72 k.Da	1/10	1.981	40-80	2:3

HRP labelling by activated peroxidase kit:

The anti-42 k.Da antibodies conjugated to HRP using the immunoPure^{R} activated peroxidase kit (Pierce, USA) did not react with the homologous *T. evansi* antigens when tested by ELISA. No colour change was observed in any of the wells reacted with the conjugated antibodies that exceeded that seen in the control wells containing only PBS/Tween (Table 6.5).

Table 6.5: ELISA absorbance values elicited by HRP-labelled anti-42 k.Da antibodies conjugated by the Pierce ImmunoPure^{R} activated peroxidase kit.

	Conjugate dilutions					
	1/10	1/20	1/40	1/80	1/160	1/320
Fraction 1	0.039	0.032	0.040	0.040	0.041	0.040
Fraction 2	0.039	0.039	0.043	0.044	0.450	0.047
Fraction 3	0.042	0.038	0.044	0.039	0.051	0.043
Fraction 4	0.044	0.042	0.045	0.043	0.041	0.043
Fraction 5	0.044	0.044	0.047	0.045	0.044	0.043
Fraction 6	0.043	0.043	0.043	0.043	0.042	0.048
Fraction 7	0.043	0.050	0.044	0.041	0.043	0.046
Fraction 8	0.049	0.046	0.049	0.046	0.047	0.045
Fraction 9	0.040	0.039	0.040	0.040	0.041	0.041
Fraction 10	0.041	0.039	0.041	0.045	0.040	0.044
PBS/T (0.046)						

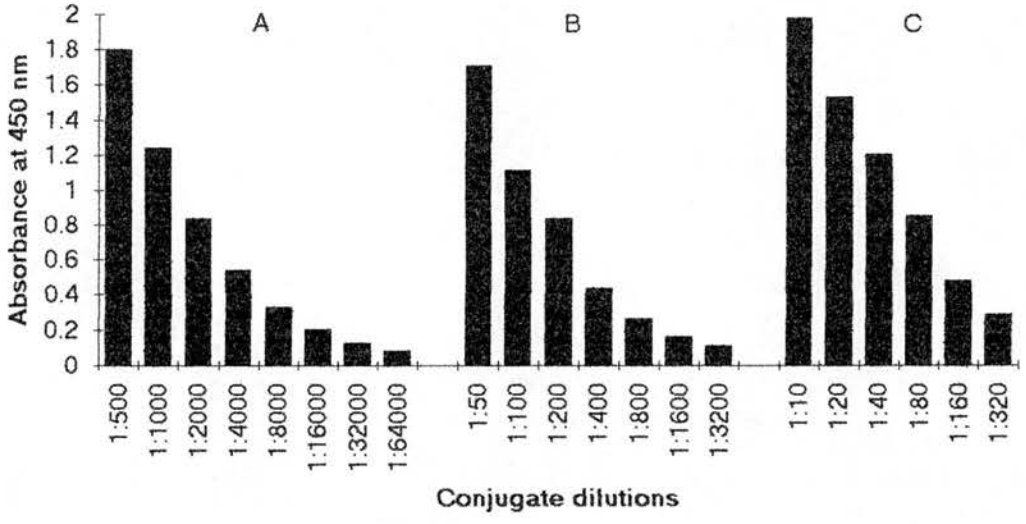
Figure 6. 1: HRP-labelled *T. evansi* antibodies coupled through the modified periodate method: Reaction to the homologous *T. evansi* population using direct ELISA.

Antigen dilution 1/80.

A: anti-42 k.Da antibodies.

B: anti-52 k.Da antibodies.

C: anti-72 k.Da antibodies.


A
B
C
Absorbance at 450 nm
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
1:500
1:1000
1:2000
1:4000
1:8000
1:16000
1:32000
1:64000
1:50
1:100
1:200
1:400
1:800
1:1600
1:3200
1:10
1:20
1:40
1:80
1:160
1:320
Conjugate dilutions

Biotinylated antibodies:

Biotinylated anti-42 and 52 k.Da IgG reacted with the homologous *T. evansi* antigen when tested by direct ELISA using an avidin-HRP conjugate. A gradual decrease was seen in the absorbance values with increasing dilution of the biotinylated antibodies (Figure 6. 2). Absorbance values obtained with the anti-42 k.Da biotinylated antibodies ranged from 2.711 at a dilution of 1/10 to 0.354 at a dilution of 1/1280 while those with the anti-52 k.Da biotinylated antibodies ranged from 2.252 to 0.165 over the same dilution range.

The biotinylated anti-42 k.Da antibodies showed an ELISA absorbance value of 1.0 at an estimated titre of 1/320 while the biotinylated anti-52 k.Da antibodies had an estimated titre of 1/160 for the same absorbance value (Table 6.6). The molar ratio of the IgG:biotin-HPDP used for each antibody was 1:4.

Table 6.6

Comparison of the activity of *T. evansi* biotinylated antibodies

Antigen	Starting dilution	Absorbance at starting dil.	Dilution at an absorb. of 1.0	antibody: biotin ratio
42 k.Da	1/10	2.711	1/320	1:4
52 k.Da	1/10	2.252	1/80-1/160	1:4

Figure 6. 2

T. evansi biotinylated antibodies: Reaction to the homologous *T. evansi* population using ELISA test utilising avidin-HRP conjugate.

Antigen dilution 1/80.

6. 4. DISCUSSION:

T. evansi antibodies in the present study were successfully separated from serum and ascitic fluid using affinity chromatography on protein A columns. The method is a well established means for the purification of IgG molecules (Tijssen, 1985, Harlow and Lane, 1988) although IgM antibodies from some species have been reported to bind to protein A (Tijssen, 1985). The conditions used in the present study, however, were likely to produce predominantly IgG antibodies particularly the high salt buffer conditions under which over 80% of IgM molecules are reported to be removed from the column with the washing buffer (Tijssen, 1985). The second eluate fraction of each antibodies in the present study contained the highest concentration of antibodies as judged by its ability to recognise the freeze-thawed *T. evansi* antigens in ELISA. This form of assay was selected to reflect the ways in which the reagents would ultimately be used for detection of antigen in serum and tissues.

The purified antibodies were successfully labelled with horseradish peroxidase using the modified periodate method (Wilson and Nakane, 1978). This is a well-established method for conjugation of the enzyme which preserves antibody reactivity by coupling to the carbohydrate portion of the enzyme and antibody molecule which is not usually involved in the antigen binding (Liddell and Cryer, 1991). Conjugates produced by this method also usually have higher activities than those produced by the two-step gluteraldehyde method with Nygren (1982) reporting five fold higher detectabilities in spot-ELISA with periodate conjugates compared with those produced with the two-step gluteraldehyde method.

The conjugates prepared from the antibodies to the42 k.Da antigen showed the highest reactivity with the *T. evansi* soluble antigen preparation. This could be due either to the greater abundance of this antigen in the soluble extract from the parasite or because the antibodies themselves were of higher affinity than those to the 52 k.Da antigen. The anti-72 k.Da McAb conjugates showed the the lowest activity of the three preparations by direct ELISA test possibly due to difference in the performance characteristics of the monoclonal and polyclonal systems (Liddell and Cryer, 1991) or differences in affinity.

Each antibody preparation was also successfully biotinylated and used in an ELISA to detect *T. evansi* antigens in association with HRP-labelled avidin. The biotin derivative used in the present study - Biotin-HPDP, has a sulfhydryl specific reactivity which preserves the immunological activity of the antibody molecule. The conjugation processes includes reduction of the immunoglobulin under mild conditions using mercaptoethylamine which cleaves the disulphides between the IgG heavy chains while preserving the disulphide linkages between the heavy and light chains. This ensures that the biotinylation site is restricted to the hinge region of the antibody leaving the antigen binding site unaffected.

The anti-42 k.Da antibodies that were directly labelled with HRP had a higher titre compared to when they were coupled to biotin. This might be due to the low proportion of antibody labelled by the biotin as the manufacturer (Pierce Chemical Company) estimates that only 57% of the IgG molecules are likely to be biotinylated using the protocol used in the present study. In the case of the anti-52 k.Da antibodies, however, no difference was observed between direct and indirect labelling, suggesting that factors other than those considered above affect binding efficiency with this form of biotin.

The Pierce immunoPure^{R} activated peroxidase kit failed to produce a functionally-labelled HRP conjugate with the anti-42 k.Da antibodies in that resulting preparation was not able to recognise the homologous *T. evansi* antigen. This failure might be due to the 10:1 enzyme:antibody ratio used in the present study. Although this ratio is within that recommended by the kit manufacturer other workers have shown that under conditions of 4 to 8-fold excess of activated enzyme the majority of antibodies remain unlabelled (Nygren, 1982). Furthermore, the amount of antibody recommended in the kit was 0.3 mg/conjugation which, depending on the purity of the antibody preparation might not be enough to recognise the antigen.

In the present study the usefulness of Protein A affinity chromatography for the separation of IgG from serum and ascitic fluid was confirmed. The resulting IgG after conjugation to HRP or biotin retained its activity and could be used in antigen-trapping ELISAs at high working dilutions for use in subsequent studies monitoring the presence of the corresponding antigens in serum and tissues of infected animals.

CHAPTER SEVEN

ANTIGEN DYNAMICS IN *TRYPANOSOMA EVANSI* INFECTION

THE AIM:

This chapter developed immunoassays and used them to investigate the dynamics of the 42, 52, and 72 k.Da *T. evansi* antigens during the course of infections in mice and rats

7. 1. INTRODUCTION:

A lot of information is known about the interaction between the host and the intact trypanosomes, in terms of the parasites distribution within the host, its infectivity and the pathology of the disease. The dynamic aspects and the fate of individual trypanosome components during infection are, however, not well understood. Such information might provide better insights into the host-parasite interaction and provide indicators of infection, progress of the disease and effectiveness of the chemotherapy. Most of the information on the behaviour of the trypanosome components during infection has come from studies designed to produce antigen detection diagnostic tests. Soluble products of trypanosomal origin have been detected in the circulation of infected animals using an antigen-capture ELISA based on a polyclonal antiserum raised against crude trypanosomal antigens (Rae and Luckins, 1984). Nantulya and Lindqvist (1989) in developing antigen-ELISA for diagnosis of bovine trypanosomiasis using monoclonal antibodies to membrane antigens of procyclic trypanosomes, detected differences in the dynamics of circulating trypanosome antigens. *T. vivax* and *T. congolense* antigens were detectable between 10 - 12 days following tsetse challenge, while *T. brucei* antigens were detectable between 8 and 14 days. Following treatment of the infected cattle with Berenil, *T. vivax* and *T. congolense*

antigens were cleared from the circulation within two weeks but the rate of clearance of *T. brucei* antigens was slower compared to the other two species. The host species has also been found to influence the dynamics of the trypanosome antigens. El Amin *et al* (1993) during an evaluation of antigen-ELISA for the diagnosis of *T. evansi* infection using the above anti-brucei group-specific antigen detected the antigens in camels 2 - 4 days after infection. In goats the parasite antigens were detected after 7 days of infection.

Two types of ELISA, competitive and non-competitive, are suitable for the detection of parasite components in body fluids (WHO, 1976, Kemeny, 1992). One form of competitive assay involves immobilising antigen onto a solid matrix and allowing it to compete with antigen present in the test sample for an enzyme labelled antibody specific to the test antigen which is added along with the test sample. If the corresponding antigen is present in the test sample, the labelled antibody is prevented from binding to the immobilised antigen and a reduced absorbance value is obtained. A disadvantage of the immobilised antigen method is that, a precise, limiting quantity of the antigen has to be immobilised. Also any dissociated antigen will compete for the labelled antibody (Kemeny, 1992). An alternative assay configuration consists of immobilised antibody and an enzyme-labelled antigen competes with any unlabelled antigen in the sample for the immobilised antibody. A disadvantage of this immobilised antibody method is that the antigen may be altered by conjugation to the enzyme and the antibody may not be able to detect it. The competitive methods are useful for quantitation of antigen present in test samples (Tijssen,1985, Kemeny, 1992).

In non-competitive assays specific antibody is immobilised onto the solid phase before the addition of test sample suspected of containing the corresponding antigen. Any antigen binding to the antibody is detected with a

conjugate consisting of the enzyme-labelled primary antibody used to coat the plate. Detectability of such assays can be increased by using a linked avidin-biotin system (Tijssen, 1985, Liddell and Cryer, 1991).

In addition to circulating in the blood trypanosome materials are also expected to be present in the tissues of the infected host and indeed they are known to inhabit extravascular sites throughout the host body (Seed and Effron, 1973, Seed *et al*, 1984, Sudarto *et al*, 1990). Enzyme immunoassays have been successfully applied to the localisation of antigens in cells or tissue sections both at the light and electron microscope level in studies on the function, development and processing of antigens in cells or tissues (Iwamasa *et al*, 1982, Leppi *et al*, 1982, Naritoku and Taylor, 1982, Mwangi *et al*, 1990,Sudarto *et al*, 1990). These methods can be used with paraffin sections from fixed tissues or cryostat sections of fresh or fixed tissues (Tijssen, 1985). A disadvantage of paraffin sections is that they do not preserve many antigens, while cryostat sections are reputed to preserve the antigens and cell structures (Harlow and Lane, 1988). The successful detection of antigens in tissue sections is influenced by three major factors. First the local antigen concentration at time of collection; second the type of tissue fixation, and third the nature of the assay (Harlow and Lane, 1988). Antigens need to be present at a high concentration for successful detection. Soluble antigens are often difficult to distinguish from background signal even when present at high concentration as they tend to diffuse in the tissues. Insoluble antigens such as membrane antigens are easy to detect because they have characteristic structures and many identical antibody-binding sites. Depending on their chemical composition antigens may be extracted or denatured by particular fixative agents such as methanol or acetone. These solvents are suitable for large proteins, but may extract some antigens. Formaldehyde denatures large

proteins and is generally more suitable for smaller antigens (Tijssen, 1985). The assay method also influences the detection of the antigens. Methods using enzyme-labelled antibodies have a higher sensitivity than the immunofluorescent methods particularly when certain procedures are used such as peroxidase anti-peroxidase (PAP) method or avidin-biotin-peroxidase complex (ABC) (Tijssen, 1985). Detection methods based on fluorochrome-labelled antibodies, however, offer a better resolution (Harlow and Lane, 1988).

The studies in this chapter describe the development and application of assays to detect *T. evansi* antigens in blood and tissues

7. 2. ASSAY DEVELOPMENT FOR *T. EVANSI* ANTIGENS SERUM AND TISSUE EXTRACTS:

7. 2. 1. BASIC ASSAY PROCEDURE: (antigen-ELISA)

A basic test procedure consisted of coating ELISA microtitre plates with an IgG preparation to either the 42 or 52 k.Da antigens. The plates were coated with a 100 µl/well of a 2-fold dilution of IgG from 1/50-1/6400, incubated overnight at 4°C and washed as described in section 2.8. Each coating dilution was then reacted with 100 µl/well of either a freezed-thawed *T. evansi* extract or normal rabbit serum using a 2-fold dilution series from 1/50-1/400. The remaining area of the plate was incubated with 100 µl/well PBS/Tween. After incubation at 37°C and washing (section 2.8), 100 µl/well of the anti-42 or 52 k.Da IgG preparations labelled with either horseradish peroxidase or biotin was added to each well

The peroxidase-labelled anti-42 and anti-52 k.Da IgG were diluted to 1/200 in PBS/Tween before use in the assay. Plates were incubated at 37°C

for 30 minutes with the conjugate and then washed before adding 100 µl/well of the TMB substrate system (section 2.8). After incubation for 15 minutes the reaction was stopped by the addition of 50µl/well of 2M sulphuric acid. The optical densities of each well was read at 450 nm using an ELISA plate reading photometer (Multiskan^{R} Plus, Labsystems Finland).

The biotinylated IgG conjugates were used at a dilution of 1/160 in PBS/Tween, incubated for 1 hour at 37^{o}C before washing. The bound conjugate was detected with either HRP-labelled avidin at a dilution of 1/10000 in PBS/Tween using the TMB substrate system, or with alkaline phosphatase-labelled avidin at a dilution of 1/5000 using 2.5 mM p-nitrophenyl phosphate (PNPP) substrate in 10 mM diethanolamine and 0.5 mM $MgCl_2$. The resulting absorbance values were read at 405 nm.

A similar assay procedure was used to establish the working dilution for the unlabelled McAb to the 72 k.Da antigen by coating the plates with unlabelled IgG in a 2-fold dilution series ranging from 1/10 to 1/1280. As before each coating dilution in 2/3 of the plate was then reacted with a 2-fold dilution series ranging from 1/10-1/1280 of the standard soluble *T. evansi* extract. The remaining third of the microtitre plate was incubated with PBS/Tween only. Antigen binding was assayed using a peroxidase labelled anti-72 k.Da IgG preparation at a dilution of 1/40. The assay was performed as described for the HRP system above.

RESULTS:

Neither the peroxidase-labelled or biotinylated antibodies to the 42 or 52 k.Da antigen gave a sufficiently strong signal that differentiated between the *T. evansi* soluble extract and normal rabbit serum (Table 7. 1).

Table 7.1 Comparison of the reaction of peroxidase-labelled and biotinylated anti-42 k.Da antibodies with *T. evansi* antigen and normal rabbit serum in antigen-ELISA

	coating antibody dilutions							
	1/50	1/100	1/200	1/400	1/800	1/1600	1/3200	1/6400
HRP-Labelled (1/200)								
antigen (1/50)	0.569	0.494	0.475	0.433	0.408	0.303	0.259	0.207
NRS (1/50)	0.560	0.457	0.450	0.450	0.416	0.310	0.213	0.172
PBS/T	0.623	0.576	0.560	0.524	0.496	0.391	0.287	0.220
Biotinylated (1/160): (avidin-HRP)								
antigen (1/50)	0.538	0.500	0.488	0.484	0.483	0.462	0.332	0.254
NRS (1/50)	0.589	0.561	0.546	0.512	0.475	0.450	0.417	0.301
PBS/T	0.544	0.534	0.528	0.514	0.489	0.446	0.392	0.297
Biotinlated (1/160): (avidin-AP)								
antigen (1/50)	0.187	0.120	0.113	0.110	0.096	0.050	0.050	0.045
NRS (1/50)	0.162	0.117	0.112	0.112	0.089	0.054	0.050	0.041
PBS/T (1/50)	0.158	0.115	0.110	0.107	0.083	0.061	0.049	0.044

The peroxidase labelled anti-72 k.Da McAb detected the homologous *T. evansi* antigen at a dilution of up to 1/320. A working dilution of up to 1/640 for the unlabelled IgG was able to react with the homologous antigen at a dilution of 1/80 (Table 7. 2).

Table 7. 2

Determination of working dilution of unlabelled McAb

	coating antibody dilutions							
	1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1280
Antigen dilution:								
1/10	1.198	1.068	1.106	1.113	1.175	1.074	1.127	1.062
1/20	0.890	0.745	0.705	0.764	0.737	0.678	0.708	0.643
1/40	0.629	0.561	0.516	0.533	0.484	0.456	0.445	0.384
1/80	0.481	0.533	0.425	0.379	0.327	0.285	0.332	0.265
1/160	0.429	0.419	0.339	0.319	0.263	0.246	0.257	0.246
1/320	0.337	0.320	0.299	0.328	0.259	0.247	0.229	0.190
1/640	0.331	0.381	0.340	0.292	0.262	0.224	0.207	0.186
1/1280	0.351	0.302	0.298	0.264	0.241	0.241	0.242	0.174
PBS/Tween: 0.196								

DISCUSSION:

The basic ELISA using anti-72 k.da McAb conjugates effectively discriminated between soluble *T.evansi* antigen and the control wells containing PBS/Tween. However, neither the anti-42 or 52 k.Da conjugates could differentiate between the *T. evansi* antigen and normal rabbit serum as similar absorbance values were obtained with antigen, normal rabbit serum and PBS/Tween irrespective of dilution of antibody or antigen.

Evidence had been previously obtained that each unlabelled antibody preparation was active against the parasite antigen under such assay conditions (see section six). The conjugated preparations were also active as evidence by its reaction with the substrate in the present basic assay and the soluble trypanosome extract should have contained enough of both the 42 and 52 k.Da antigens to be detected by the antibodies.

The failure of this particular assay system is attributed to high absorbance values with the negative control system rather than lack of reactivity with the target antigen. Such results could arise from the binding of normal rabbit serum to the unlabelled IgG or the binding of the conjugate to the unlabelled IgG. Such non-specific reactions are known to increase background levels in the such assays and adversely affect its discriminatory power (Tijssen, 1985). These non-specific reactions can be due to a number of mechanisms (Tijssen, 1985, Tsang et al, 1985) and number of different approaches have been used to reduce their impact on assays. These include

a)Adsorption of labelled antibody to the solid matrix that can result in high background levels. This can be eliminated or reduced by blocking the remaining binding sites of the matrix using blocking agents such as bovine serum albumin (Towbin et al, 1979, Aubertin et al, 1983), b)The presence of auto-antibodies (rheumatoid factors) in the assay complex (Tijssen, 1985) which react specifically with IgG or IgM of the same species giving rise to false-positive reactions. The reaction of the normal rabbit serum with the unlabelled IgG and that of the labelled and unlabelled IgG in the present study could possibly be due to these rheumatoid factors. Since the Fc fragment of antibody is the only part of the molecule that carries receptors for rheumatoid factors (Stanworth and Turner, 1978), a number of approaches have been used to reduce or eliminate interference by rheumatoid factors such as the use of conjugates based on the F(ab)2 fragment (Tijssen, 1985). Rheumatoid factors can also be removed with an excess of Fc fragments (Tijssen, 1985) or more easily by pre-incubation with small amount (1-2%) of normal serum from the same species (Kemeny, 1992).

Further studies were, therefore, carried out to determine the source of the non-specific reactions obtained with the basic assay procedure in an attempt to improve the overall performance of the antigen-trapping system.

7. 2. 2. Improving the basic assay:

A number of factors influencing the assay system were investigated with the aim of improving the discriminating power of the assay. These were

all carried out initially with anti-42 k.Da IgG preparation and promising modifications applied to the anti-52k.Da preparation.

(a) Blocking the binding sites of the plate with bovine serum albumin (BSA):

A blocking step in which 100 μl per well of 1% BSA (w/v) was introduced in the above basic assay with the aim of blocking any remaining binding sites on the microtitre plates after incubation with the unlabelled IgG. The remainder of the assay was performed as previously described for the peroxidase-labelled IgG and TMB system.

(b) Incorporation of normal rabbit serum (NRS) in the conjugate dilution buffer:

1% (v/v) normal rabbit serum was added to PBS/Tween that was used for preparation of working dilution of the peroxidase-labelled or biotinylated anti-42 IgG conjugates. In this study the plates were coated with the anti-42 IgG at a dilution of 1/50 in the carbonate/bicarbonate buffer, incubated and washed as described previously. The soluble *T. evansi* antigens and NRS in PBS were used at a two-fold dilution series ranging from 1/50 to 1/6400 and the conjugated antibodies were used at a dilution range from 1/200 to 1/3200 in PBS/Tween containing 1% NRS.

The addition of 1% NRS to the conjugate dilution buffer was also undertaken with the anti-52 k.Da antibodies. Dilution ranges investigated in this case were as used in the basic assay described in section 7.2.1 with the peroxidase-labelled and biotinylated anti-52 k.Da antibodies diluted in PBS/Tween containing 1% (v/v) NRS.

(c) Salt precipitation of immunoglobulins from serum:

Saturated ammonium sulphate (SAS) (1000 g / 1 litre of distilled water) was used to precipitate the immunoglobulins in the serum raised against the 42 k.Da antigen. A two ml serum sample was diluted 1:2 in ice-cold PBS and 4.92 ml of SAS was added dropwise to give a final concentration of 45% saturation (v/v). The mixture was stirred at 4°C for 30 minutes, and centrifuged at 1000 g for 15 minutes at 4°C. The resulting precipitate was washed by centrifugation with 45% SAS as above. It was then re-dissolved in 2 ml of PBS and centrifuged at 5000 g for 15 minutes at 4°C to remove any insoluble materials. The immunoglobulin (Ig) in the supernatant was re-precipitated as above using 1.34 ml of SAS to give a final concentration of 40% saturation and then centrifuged at 1000 g, 15 minutes, at 4°C. The precipitate was redissolved in 0.5 ml PBS and dialysed against five litres PBS overnight at 4°C. The dialysed Ig solution was finally centrifuged at 5000 g, 15 minutes, at 4°C to remove any insoluble materials. The protein content of the Ig containing supernatant was measured spectrophotometrically at 280 nm and the Ig solution was stored at -20°C.

The immunoglobulin preparation was then coupled to HRP using the modified periodate method as described in section 6 .3.

The labelled and unlabelled SAS precipitated immunoglobulin preparations were then used in the basic assay with the SAS precipitated preparations replacing the protein A separated preparations.

(d) $F(ab)_2$-HRP conjugate:

$F(ab)_2$ fragment was prepared from the protein A separated anti-42 k.Da IgG using ImmunoPure^R $F(ab)_2$ Preparation Kit (Pierce, USA) and following the manufacturers instructions involving pepsin digestion of the IgG

molecule. This fragment was then coupled to HRP using the modified periodate method as described in section 6.2.2.

The activity of the $F(ab)_2$ preparation was tested in the basic assay by coating the microtitre plates with the anti-42 k.Da $F(ab)_2$ fragment at a dilution of 1/50 in the carbonate /bicarbonate buffer. The *T. evansi* soluble materials and normal rabbit serum in PBS were used at a two-fold dilution range from 1/25 to 1/400. The antigen-antibody complex was detected with the anti-42 k.Da $F(ab)_2$-HRP conjugate at dilutions ranging from 1/50 to 1/6400 in PBS/Tween.

RESULTS:

The introduction of a blocking step with 1% BSA did not improve the performance of the basic assay as assay continued to fail to differentiate between the antigen and NRS. Results for the antigen and NRS at 1/50 dilution is shown in table 7.3.

The inclusion of 1% normal rabbit serum in PBS/Tween, as a blocking agent in the buffer used to dilute the HRP-labelled or biotinylated anti-42 k.Da antibodies, however, substantially reduced the absorbance with the NRS and PBS/Tween. It enabled the assay to differentiate between the antigen and NRS (Table 7.4).

Table 7.3

The effect of a BSA blocking step, on assay performance

	Coating antibody dilutions							
	1/50	1/100	1/200	1/400	1/800	1/1600	1/3200	1/6400
antigen (1/50)	0.570	0.559	0.553	0.540	0.398	0.359	0.319	0.323
NRS (1/50)	0.609	0.587	0.576	0.560	0.406	0.324	0.377	0.310
PBS/T	0.523	0.525	0.517	0.508	0.467	0.371	0.311	0.315

Table 7.4

The effect of using PBS/T containing 1% normal rabbit serum as a dilution buffer for the antibody conjugates on the assay performance

	Conjugated antibody dilutions				
	1/200	1/400	1/800	1/1600	1/3200
HRP-Labelled:					
antigen (1/50)	1.048	0.577	0.400	0.306	0.136
NRS (1/50)	0.286	0.176	0.116	0.089	0.063
PBS/T	0.203	0.118	0.097	0.087	0.074
Biotinylated:					
antigen (1/50)	1.231	0.858	0.624	0.392	0.265
NRS (1/50)	0.458	0.338	0.260	0.180	0.119
PBS/T	0.303	0.254	0.218	0.172	0.126

Similar results were also obtained with the 52 k.Da antibodies (Table 7.5).

Table 7.5 Dilution of labelled anti-52 k.Da antibodies with PBS/Tween containing 1% NRS

	coating antibody dilutions							
	1/50	1/100	1/200	1/400	1/800	1/1600	1/3200	1/6400
HRP-Labelled (1/200)								
antigen (1/50)	0.994	0.900	0.763	0.652	0.656	0.640	0.459	0.460
NRS (1/50)	0.294	0.247	0.178	0.159	0.142	0.134	0.106	0.103
PBS/T	0.290	0.288	0.179	0.151	0.126	0.138	0.105	0.117
Biotinylated (1/160): (avidin-HRP)								
antigen (1/50)	1.723	1.536	1.543	1.714	1.599	1.542	0.561	0.573
NRS (1/50)	0.290	0.222	0.235	0.287	0.291	0.294	0.290	0.290
PBS/T	0.266	0.264	0.254	0.265	0.289	0.290	0.288	0.289

Immunoglobulin purified by salt precipitation produced reduced absorbance readings with both antigen and NRS compared to those obtained with the same antibody purified with protein A chromatography. The assay, however, did not differentiate between the antigen and NRS (Table 7.6).

Table 7. 6 The effect of immunoglobulin purification method (SAS precipitation) on the assay performance

	Coating antibody dilutions							
	1/50	1/100	1/200	1/400	1/800	1/1600	1/3200	1/6400
antigen (1/50)	0.252	0.231	0.238	0.241	0.250	0.292	0.255	0.251
NRS (1/50)	0.212	0.190	0.192	0.205	0.231	0.253	0.222	0.199
PBS/T	0.254	0.233	0.251	0.256	0.296	0.317	0.304	0.276

The assay using HRP anti-42 k.Da $F(ab)_2$ fragment produced significant signal strength to differentiate between the antigen and NRS. A binding ratio of 3.0 was obtained at a 1/100 dilution of $F(ab)_2$ (Table 7.7).

Table 7. 7 The effect of $F(ab)_2$-HRP on reduction of non-specific reaction

	$F(ab)_2$-HRP dilutions							
	1/50	1/100	1/200	1/400	1/800	1/1600	1/3200	1/6400
antigen (1/50)	1.108	0.732	0.392	0.192	0.119	0.077	0.060	0.048
NRS (1/50)	0.457	0.272	0.129	0.087	0.063	0.049	0.046	0.045
PBS/T	0.402	0.274	0.160	0.092	0.069	0.065	0.047	0.047

DISCUSSION:

In the present assay the addition of 1% normal rabbit serum to the conjugate dilution buffer increased the binding ratio with the antigen essentially by reducing the absorbance of the NRS control. Consequently, the assay was able to discriminate between the antigen and NRS. The only other modification to the assay that had a beneficial effect on the discriminatory

power of the assay was the use of $F(ab)_2$ fragments rather than whole IgG molecule preparations. These results suggests that the presence of rheumatoid factors causing cross-reactions between the labelled antibody and coating IgG was the major cause of the failure of the basic assay to discriminate between antigen and normal rabbit serum. This type of non-specific-reactions have been shown to be neutralised by pre-incubation with small amount of serum (1-2%) from the cross reacting species (Kemeny, 1992). 1% normal serum have been used in corporation with PBS/T for dilution of antibody conjugates to reduce or eliminate non-specific reaction in antigen-ELISAs (Nantulya *et al*, 1989; Nantulya and Lindqvist, 1989). The presence of normal serum in the conjugate diluent should help to neutralise the effect of rheumatoid factors by binding to the offending antibody. Similarily, the use of $F(ab)_2$ fragment reduced non-specific reactivity. This fragment does not carry receptors for rheumatoid factors (Stanworth and Turner, 1978) hence the use of $F(ab)_2$ fragment should eliminate the effect of these rheumatoid factors.

Both of the modifications used in this study that increased the absorbance values of the antigen-antibody reaction, therefore, appeared to be associated with rheumatoid-factor activity. The effect possibly arising from the coating antibody competing with the antigen for the labelled antibody in the assay in the absence of NRS or $F(ab)_2$ fragment. These modifications probably served to increase the number of labelled antibody molecules binding only to the antigen with strong avidity compared to those with the coating IgG, hence increasing the absorbance level in the antigen-containing wells. The reduction in the absorbance values before the assay modification was possibly due to the dissocciation of the bond between the conjugate and the coating IgG during washing as these cross-reactants usually bind less avidly than the specific antigens (Tijssen, 1985).

The inclusion of a BSA blocking step in the assay did not improve the discriminatory power of the basic assay. This result suggests that non-specific binding of the antigen and/or the labelled antibody to the wells of the microtitre plate was not responsible for the increased level of background. This is probably linked to the presence of Tween 20 in PBS for all wash solutions and diluents which is an effective a blocking agent that inactivates the binding sites of the matrix (Mohammad and Esen, 1989).

Possible interactions between the IgG preparations arising from their isolation using protein A matrix were discarded as the substitution of ammonium sulphate precipitated immunoglobulins did not improve the discriminating power of the assay.

Consequently, in all further studies 1% NRS was included in the diluent for appropriate antibody conjugate.

7. 2. 3. Comparison of the Sensitivity of Different Conjugates for Antigen Detection:

The sensitivity of both peroxidase-labelled and biotinylated anti-42 and 52 k.Da antibodies was compared using the modified antigen capture-ELISA by titration against *T. evansi* soluble extract.

In this part of the study the basic ELISA assay as described in section 7.2.1 was performed in which microtitre plates were coated with unlabelled IgG to the 42 or 52 k.Da antigen diluted to 1/50 or 1/200. The coated plates were then titrated with the *T. evansi* soluble extract along with NRS as control over a 2-fold dilution range from 1/50 to 1/6400 PBS. The equivalent labelled IgG preparation was then added to all wells at a dilution of 1/200 in PBS/Tween containing 1% (v/v) NRS.

Conjugates preparations tested in this way were a) HRP-labelled anti-42 and 52 k.Da IgG, b) biotinylated IgG to 42 and 52 k.Da with avidin coupled

to HRP or alkaline phosphatase. Results were expressed as an antigen titre, equivalent to the last antigen dilution that showed an absorbance of at least twice that of the NRS at the same dilution.

RESULTS:

The biotinylated anti-42 k.Da antibodies utilising the avidin-HRP detection system produced the highest antigen end-point titre of 1/400 along with the highest antigen binding ratios (Table 7.8). The peroxidase-labelled antibodies had an slightly lower end-point titre (1/200) and antigen binding ratios, while the lowest performance indicators were seen with the biotin-avidin alkaline phosphatase system with an end-point titre of 1/50 and a binding ratios close to 2.0 (Table 7.8). Similarly, in case of the 52 k.Da antigen system the biotin-avidin HRP had the highest antigen end-point titre and antigen binding ratios (Table 7.8).

Overall the anti-52 k.Da conjugates appeared to be able to detect the *T. evansi* soluble extract at higher dilutions than the anti-42 k.Da conjugates.

Table 7. 8

Comparison of sensitivity of the conjugated anti-42 and 52 k.Da antibodies

	Antigen end-point titre	Absorbance at end-point titre		
		Antigen	NRS	Antigen binding Ratio
Anti-42 k.Da conjugates:				
Biotin-avidin-HRP:	1/400	0.985	0.271	3.6
Biotin-avidin-AP:	1/50	0.239	0.115	2.1
HRP conjugate:	1/200	0.419	0.160	2.6
Anti-52 k.Da antibodies:				
Biotin-avidin-HRP:	1/800	1.238	0.385	3.2
Biotin-avidin-AP:	1/400	0.349	0.138	2.5
HRP conjugate:	1/400	0.534	0.246	2.2

DISCUSSION:

The results from the present study have confirmed the ability of the antibodies raised to the 42 or 52 k.Da antigens to detect their corresponding antigens when the antibodies are coupled either directly to HRP or used in a biotin-avidin system. In the present study the biotin-avidin system utilising HRP as the enzyme conjugate was found to be the most sensitive detection system as it could detect lower concentration of antigen as judged by the results of end-point titration of antigen. This is probably related to the large number of label molecules that are attached by to the antibody when compared to the directly labelled systems and increases detectability (Liddell and Cryer, 1991, Tijssen, 1985, Johnstone and Thorpe, 1982).

The anti-52 k.Da antibodies in the present study detected the *T. evansi* crude antigen at greater dilutions than those raised against the 42 k.Da antigen although the concentration of the 52 k.Da antigen was lower than that of 42 k.Da as determined by densitometric analysis of SDS-PAGE preprations (results not shown). This suggests that the 52 k.Da antibodies had a higher affinity for the antigen than the equivalent ones to the 42 k.Da antigen.

The biotinylated antibodies to the 42 or 52 k.Da antigen utilising avidin coupled to horseradish peroxidase were consequently chosen for the development of assays for the detection of their corresponding antigens in blood and tissues.

7. 2. 4. Evaluation of the developed assay and development of an immunohistochemical test:

After the establishment of the modified ELISA assay under *in vitro* conditions the assay was subsequently evaluated for its ability to detect the antigens *in vivo* in rabbits and mice.

[a] Evaluation of the ELISA test:

(i) Materials and Methods:

Two sources of antigen were utilised in this assay, serum and tissue extracts.

(a) Serum:

A rabbit was inoculated intravenously with 10 mg of freeze-thawed *T. evansi* soluble extract, and bled for serum one day before inoculation, at 0.5, 1, 2, 4, and 24 hours post-inoculation, and then daily until the end of the experiment 6 days later. The serum was prepared as described in section 2.6.

(b) Tissue extracts:

Two mice were infected intraperitoneally with $1x10^6$ trypanosomes TREU 2165. On day 4 post-infection, the organs of one of the infected mice as well as a non-infected control mouse were perfused as described below and a number of their organs collected and used for the preparation of tissue extracts specifically, the organs collected were spleen, liver, kidneys, brain, heart, and lungs. At the same time remaining infected mouse was treated with Berenil at a dose rate of 75 μg/mouse and its organs was perfused 10 days later.

Organ perfusion was carried out according to the method of Mulumba and Wery (1983) as modified by Turner *et al* (1986). In this method the posterior and anterior venae cavae were cut, and heparinised PSG was injected into the left ventricle. Perfusion was continued for a further 20 ml after the kidneys became fully blanched.

The protocol used for the preparation of the tissue extracts was adapted from that of Ekejindu *et al* (1986) and Dignam (1990). Briefly, tissue samples from each organ were washed several times in cold PBS before they were cut into small pieces in PBS using forceps and scissors. Further disruption of the

tissues was achieved by pushing the tissue through a coarse sieve with the blunt edge of the forceps. Large tissue particles were removed by centrifugation at 15000 g for 30 minutes at 4°C and the supernatant was carefully removed, aliquoted and stored at -20°C, or assayed directly.

(c) Assay procedure:

The basic assay was performed by coating ELISA microtitre plates overnight at 4°C with the unlabelled anti-42, 52 or 72 k.Da antibodies at a dilution of 1/50, 1/200 or 1/640 respectively. Each antibody was then reacted with 100 µl/well of undiluted serum collected from the rabbit before and at different times after inoculation with *T. evansi* soluble extract or tissue extract from normal or infected mice diluted to 1/16 in PBS/T. Biotinylated anti-42 or 52 k.Da antibodies at a dilution of 1/200, or HRP-labelled anti-72 k.Da McAb at a dilution of 1/40 were then added to each well and the assay was performed as described previously (section 7.2.1). Serum samples and tissue extracts showing absorbance values more than twice that of the pre-inoculation serum from the rabbit or normal mouse tissues were considered positive for *T. evansi* antigens.

[B] Immunohistochemical detection of antigens in tissues:

(i) Materials and Methods:

(a) Preparation of cryostat sections:

Samples of organs from the infected and control mice were frozen at -70°C immediately after being taken from these animals. They were trimmed frozen and mounted onto cold specimen holders using OCT embedding compound (Tissue-Tek, Miles Labs., Ltd.) and 8 µm thick sections were cut at -42°C using a cryostat microtome (Bright Instrument Company Ltd.). The sections were mounted on 8 well multispot slides (C.A. Hendley, (Essex),

Ltd.) which had previously been dipped into a chrome alum gelatine solution (Appendix 24) and dried overnight at 37°C.

The sections were allowed to dry for 2 - 3 hours at 37°C and were then fixed in cold acetone at -20°C for 30 minutes and left to dry for 10 minutes. The sections were rinsed once in PBS for 10 minutes, kept frozen or used directly in the assay.

(b) Assay procedure:

Any endogenous peroxidase in the tissue sections was inactivated by incubation for 10 minutes in 70% (v/v) ethanol, in methanol containing 1% (v/v) hydrogen peroxide for 10 minutes, and for a further 10 minutes in 70% ethanol. The sections were then dried and rinsed once in PBS for 15 minutes. Sections from each organ were then incubated for one hour at room temperature under moist conditions with 50 µl of the biotinylated antibodies to the 42 or 52 k.Da antigen applied as a 2-fold dilution range from 1/10 - 1/40 or peroxidase-labelled McAb to the 72 k.Da antigen applied over a 2-fold dilution range from 1/5 to 1/20.

Unreacted antibodies were removed by washing the sections in three changes of PBS of 5 minutes each, and excess buffer was removed with a tissue paper around the edges of the slides. Each section containing the biotinylated antibodies received 50 µl of avidin-HRP at a dilution of 1/200, incubated and washed as above. 50 µl of 3,3/-diaminobenzidine substrate (Sigma Fast™ DAB tablets) dissolved in distilled water were then added to all sections and incubated for 15 minutes. The sections were then washed as before and counter-stained by dipping in aqueous hematoxylin for 10 seconds. The sections were then washed in distilled water for 5 minutes and dehydrated in graded concentrations of ethanol: twice in 70%, 90% and twice in 100%. The sections were cleared twice in Histo-clear™ (National Diagnostics, New

Jersey, USA), mounted in DPX and examined for the presence of antigen-antibody complex under light microscope. The dehydration and clearing steps were applied for 30 seconds each.

Controls for the specificity of the test include the replacement of the conjugated anti-*T. evansi* antibodies with normal rabbit serum /or PBS, and equivalent of tissues from uninfected mice.

RESULTS:

(i) T. evansi antigens in the circulation of the inoculated rabbit:

The anti-42 k.Da *T. evansi* antibodies detected antigens in the blood of the rabbit by 2 hours after inoculation. Thereafter, there was a gradual decline in the antigen titre which reached pre-inoculation level at 72 hours post-inoculation (Figure 7. 1). Similarly, the 52 k.Da antigen was also detected at 2 hours after inoculation and gradually declined to the pre-inoculation level by 72 hours post-inoculation (Figure 7. 2). The 72 k.Da antigen, however, reached a detection level at one hour post-inoculation and gradually declined to pre-inoculation level 24 hours post-inoculation (Figure 7. 3).

Figure 7. 1

The dynamics of the 42 k.Da antigen in the circulation of rabbit inoculated with *T. evansi* crude soluble materials.

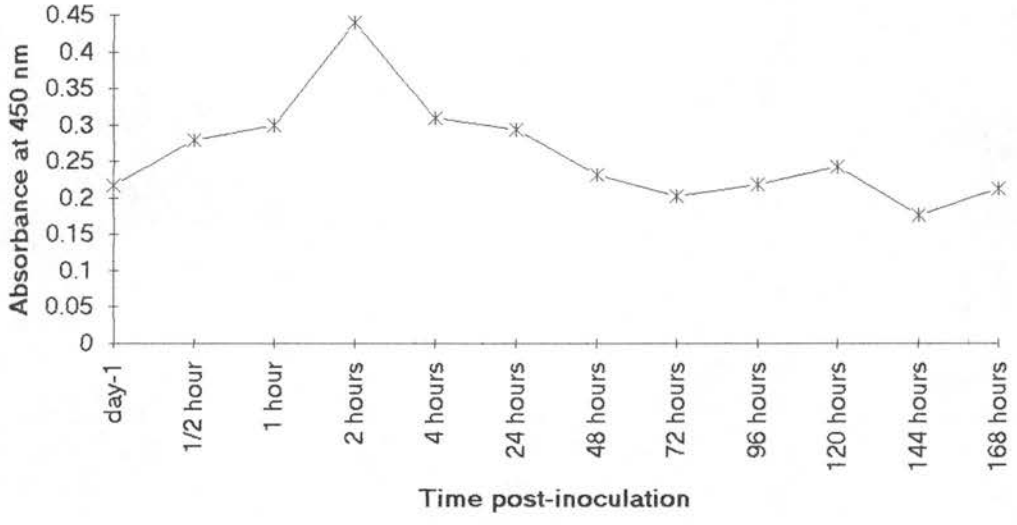

Absorbance at 450 nm
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
day-1
1/2 hour
1 hour
2 hours
4 hours
24 hours
48 hours
72 hours
96 hours
120 hours
144 hours
168 hours
Time post-inoculation

Figure 7. 2

The dynamics of the 52 k.Da antigen in the circulation of rabbit inoculated with *T. evansi* crude soluble materials.

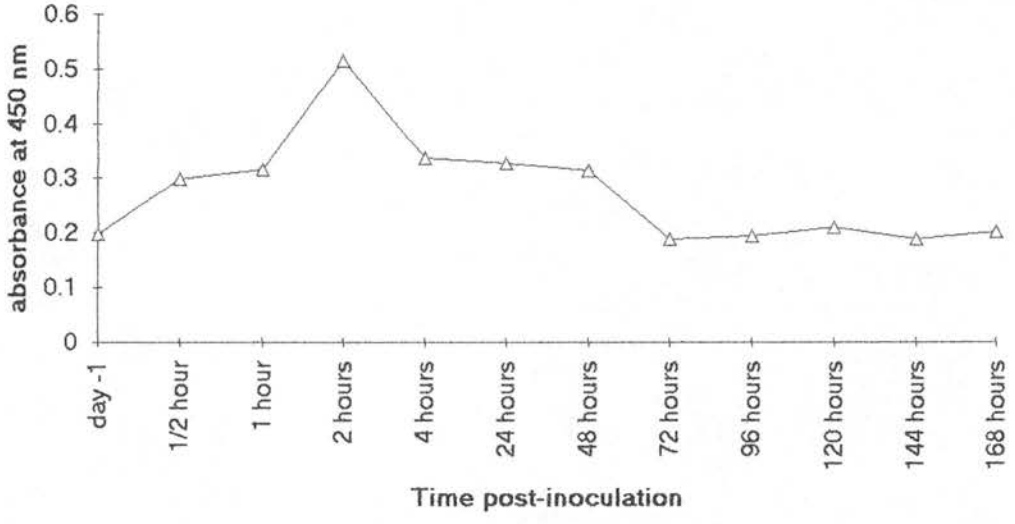
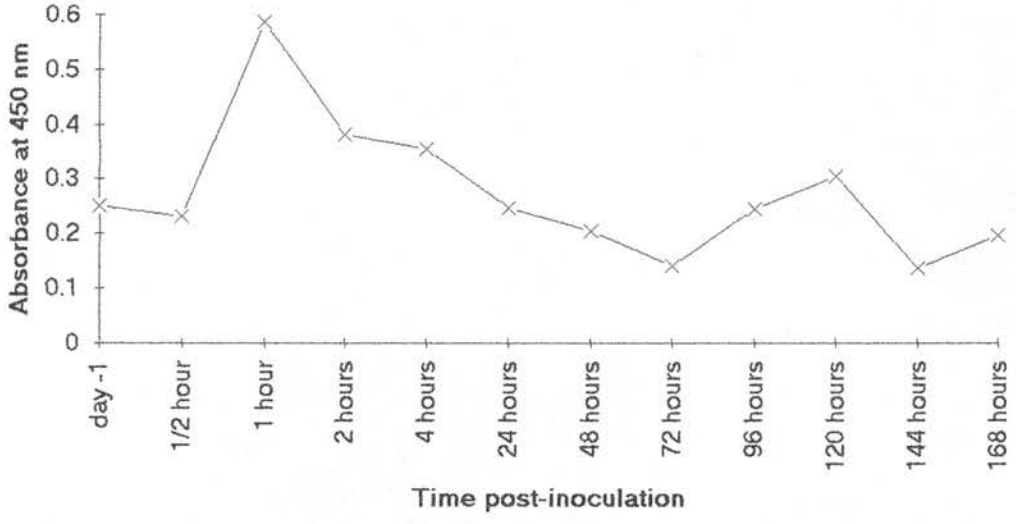

absorbance at 450 nm
0.6
0.5
0.4
0.3
0.2
0.1
0
day -1
1/2 hour
1 hour
2 hours
4 hours
24 hours
48 hours
72 hours
96 hours
120 hours
144 hours
168 hours
Time post-inoculation

Figure 7. 3

The dynamics of the 72 .k.Da antigen in the circulation of rabbit inoculated with *T. evansi* crude soluble materials.


Absorbance at 450 nm
0.6
0.5
0.4
0.3
0.2
0.1
0
day -1
1/2 hour
1 hour
2 hours
4 hours
24 hours
48 hours
72 hours
96 hours
120 hours
144 hours
168 hours
Time post-inoculation

(ii) *T. evansi* antigens in tissue extracts of infected mice:

The antibodies to 42 k.Da antigen detected the antigen in tissue extracts collected from the spleen and brain of the infected mice at 4 days after infection (Table 7. 11). By 14 days post-infection this antigen was only detectable in the kidneys.

Evidence of the presence of 52 k.Da antigen was not detected in any of the organs extracts at 4 days after infection (Table 7. 11), but by 14 days post-infection evidence of the antigen was found in tissue extracts from kidneys, brain, heart and lungs (Table 7. 11).

The 72 k.Da antigen was not detected in any of the tissue extracts examined (Table 7. 11).

Table 7. 11

Absorbance values of *T. evansi* antigens in tissue extracts of the experimentally infected mice.

	42 k.Da		52 k.Da		72 k.Da	
	normal	infected	normal	infected	normal	infected
At 4 days:						
spleen	0.261	1.024*	0.301	0.335	0.199	0.200
liver	0.809	0.991	0.805	0.824	0.233	0.391
kidney	0.245	0.411	0.242	0.301	0.165	0.181
lung	0.270	0.486	0.290	0.442	0.163	0.204
heart	0.261	0.513	0.270	0.380	0.172	0.183
brain	0.265	0.753*	0.267	0.421	0.178	0.195
At 14 days:						
spleen	0.251	0.488	0.303	0.564	0.196	0.203
liver	0.801	0.974	0.808	0.820	0.238	0.357
kidney	0.244	0.503*	0.240	0.569*	0.162	0.172
lung	0.262	0.440	0.286	0.645*	0.169	0.170
heart	0.259	0.378	0.274	0.550*	0.174	0.182
brain	0.267	0.457	0.265	0.575*	0.182	0.265

* ≡ positive reaction (more than twice that of uninfected control).

(iii) *T. evansi* antigens on cryostat sections:

None of the three antigens were detected in the cryostat sections prepared from the organs collected from the infected mice on day 4 or 14 post-infection (Table 7. 12).

Table 7. 12

T. evansi antigens detected in tissue extracts and cryostat sections from mice experimentally infected with *T. evansi*

	42 k.Da antigen		52 k.Da antigen		72 k.Da antigen	
organ	extract	section	extract	section	extract	section
spleen	+ *	-	-	-	-	-
liver	-	-	-	-	-	-
kidney	+ **	-	+ **	-	-	-
brain	+ *	-	+ **	-	-	-
heart	-	-	+ **	-	-	-
lung	-	-	+ **	-	-	-

N.B.

*: 4 days after infection.

* *: 14 days after infection.

7. 2. 5. Antigen dynamics in *T. evansi* infected rats:

(i) Materials and Methods:

(a) Experimental animals:

Ten male Wistar rats (Bantin & Kingman Ltd. UK) were used in this experiment, 4 of which served as un-infected controls.

(b) Infection protocol:

Six rats were infected intraperitoneally with $1x10^4$ *T. evansi* (TREU 2165) trypanosomes per rat. Parasitaemia was monitored daily until the end of the experiment on day 17 post-infection using the microhaematocrit centrifugation technique. Tail blood was collected daily from the infected and control rats as a source of serum. All infected rats were treated with Berenil on day 8 post-infection. On day 12 after infection one of the infected rats was sacrificed, perfused as described previously and spleen, liver, kidney, lung, heart and brain removed for preparation of tissue extracts and cryostat sections. These materials were also prepared at the end of the study from surviving infected and control rats.

(c) Detection of antigens in circulation and tissue extracts:

The antigen-ELISA developed in the previous section was used to monitor levels of the 42, 52, and 72 k.Da antigens in the blood and tissues of the infected rats. Pooled serum collected from the each infected or uninfected rats was diluted to 1/3 before use in the assay, while tissue extracts were diluted to 1/16 in PBS/T before use. Serum and tissue extracts samples showing an absorbance value greater than twice that from uninfected control animals were considered positive for the antigens.

(d) Detection of antigens on cryostat sections:

Cryostat sections prepared from the organs collected from both infected and uninfected rats were examined for the presence of *T. evansi* antigens using the immunohistochemical technique described in the previous section. The

biotinylated antibodies to the 42 and 52 k.Da antigens were used at a dilution of 1/20 and the HRP-labelled McAb to the 72 k.Da at a dilution of 1/10 and the assay performed as described previously.

RESULTS:

(i) Parasitaemia:

The parasites were first detected in the tail blood of the rats at 4 days after infection. Thereafter, their number increased reaching a peak on day 8 post-infection. Following treatment with Berenil on day 8, the parasites disappeared from the circulation the next day and were not detected up to the time that the experiment was terminated at 17 days post-infection (Table 7. 13).

(ii) Antigenaemia:

The antibodies to the 52 k.Da antigen detected the antigen in the blood of the rats by day 6, reaching peak activity on day 8 post-infection. After treatment on day 8 the activity declined reaching negative control level on day 11 post-infection and the rats remained antigen-negative until the end of the experiment 17 days after infection (Table 7. 13).

The 42 k.Da antigen activity was first detected on day 7 after infection reaching a peak activity on day 8 post-infection. After treatment on day 8, there was a gradual decrease in the activity reaching negative control levels by day 11 post-infection that was maintained until the end of the experiment 17 days after infection. (Table 7. 13).

The presence of the 72 k.Da antigen was first detected on day 8 post-infection. Again, after treatment, there was a sharp decline in the activity level reaching negative control level by the next day that was maintained until the end of experiment 17 days after infection.(Table 7. 13).

[C] Antigens in Tissues:

The 42 k.Da antigen was detected in the spleen and kidneys of the infected rats when extracts of tissues collected at the end of the experiment 17 days after infection were examined by antigen-ELISA (Table 7. 14). This antigen was not detected in any tissue extracts collected one day following the disappearance of the antigen from the circulation. The 52 k.Da was detected only in the spleen extract prepared 17 days after infection (Table 7. 14). The 72 k.Da antigen was not detected in any of the tissue extracts examined (Table 7. 14).

None of the antigens were detected in the sections prepared from the infected rats one day after the disappearance of the antigens from the blood circulation or at the end of the experiment 17 days post-infection.

Table 7. 13

Parasitaemia and antigenaemia in the experimental rats

days after infect.	tryps per 20 fields	42 k.Da O.D*	control serum O.D*	52 k.Da O.D*	control serum O.D*	72 k.Da O.D*	control serum O.D*
1	0	0.212	0.210	0.257	0.189	0.154	0.150
2	0	0.360	0.216	0.286	0.249	0.149	0.140
3	0	0.379	0.209	0.249	0.220	0.150	0.144
4	10	0.350	0.260	0.286	0.215	0.146	0.131
5	22	0.344	0.242	0.283	0.231	0.150	0.148
6	41	0.551	0.291	0.562	0.255	0.174	0.141
7	85	0.586	0.292	0.894	0.259	0.294	0.153
8	200	1.151	0.301	1.346	0.265	0.492	0.192
9	0	1.004	0.211	1.078	0.212	0.267	0.190
10	0	0.919	0.258	1.159	0.234	0.280	0.176
11	0	0.486	0.263	0.407	0.239	0.251	0.183
12	0	0.350	0.280	0.401	0.234	0.231	0.173
13	0	0.397	0.225	0.432	0.238	0.242	0.164
14	0	0.361	0.220	0.417	0.212	0.248	0.191
15	0	0.357	0.262	0.421	0.221	0.297	0.190
16	0	0.354	0.216	0.397	0.207	0.260	0.186
17	0	0.264	0.205	0.292	0.209	0.281	0.188

* O.D. values represents a mean of 2 tests, and values more than double that of the negative control were considered positive.

Table 7. 14

Absorbance values of *T. evansi* antigens in tissue extracts of the experimentally infected rats.

	42 k.Da		52 k.Da		72 k.Da	
	uninfected	infected	uninfected	infected	uninfected	infected
At 12 days:						
spleen	0.350	0.352	0.320	0.376	0.327	0.377
liver	0.702	0.794	0.520	0.543	0.605	0.611
kidney	0.215	0.289	0.274	0.399	0.404	0.441
lung	0.281	0.350	0.367	0.383	0.374	0.415
heart	0.298	0.306	0.345	0.388	0.401	0.418
brain	0.220	0.233	0.316	0.381	0.412	0.466
At 17 days:						
spleen	0.350	0.732*	0.320	0.673*	0.327	0.352
liver	0.702	0.774	0.520	0.551	0.605	0.624
kidney	0.215	0.456*	0.274	0.392	0.404	0.415
lung	0.281	0.292	0.367	0.374	0.374	0.395
heart	0.298	0.308	0.345	0.346	0.401	0.407
brain	0.220	0.259	0.316	0.373	0.412	0.421

* ≡ positive reaction (more than twice that of the uninfected control).

DISCUSSION:

The results obtained in this part of the study have confirmed the ability of the antigen capture-ELISA developed earlier to detect *T. evansi* antigens in serum and tissues. When soluble extracts of the parasite were inoculated in an aqueous form intravenously into a rabbit all three antigens were detected between 1 to 2 hours post-inoculation due to their circulation with blood throughout the animal body. Unaggregated protein antigens are known to distribute evenly throughout the animal›s blood before being cleared by the phagocytic cells (Tizard, 1992). Thereafter, the level of the antigens declined between 1 to 3 days after inoculation, either as result of dilution or as a result of phagocytic clearence.

Of the three antigens included in the present study, the 52 k.Da antigen was the first antigen to be detected in the circulation of *T.evansi*- infected rats. Such early appearance might be allied with the antigen's surface-association making it one of the first antigens that would be released from the parasites early during infection. The 42 k.Da antigen was the next antigen to be detected followed by the 72 k.Da fraction. Although the level of the 42 k.Da and the 52 k.Da antigens remained high for two days following treatment a sharp decline was seen on the second day. Conversely, the 72 k.Da antigen was rapidly cleared from the circulation following treatment. The rapid clearance of these antigens from the circulation suggests that they could have been circulating as antigen-antibody complexes. Such complexes are known to be rapidly cleared from the circulation by phagocytic cells (Tizard, 1992) and elimination of antigens from the circulation by these mononuclear phagocytic cells is inefficient when antibodies have low affinity for the antigen (WHO, 1977).

The peak level of antigenaemia generally coincided with the highest levels of parasitaemia. It is likely that the ELISA result reflects the presence of free target antigens in the circulation of the infected rats rather than antigen appearing in the blood sample due to breakdown of parasites after blood sampling. Previous studies had shown that trypanosomes at a concentrations of approximately $1x10^9$ trypanosomes/ml would be needed in the blood sample to reach the absorbance levels obtained with serum from the rats. These levels were never reached during the course of infection in the present study.

In tissue extracts the 42 k.Da antigen was detected in the brain and spleen of the infected mice during early infection, while the 52 and the 72 k.Da antigens were not detected in these tissues during early stages of infection either due to their scarcity or differences in processing the individual trypanosomes components. Following two weeks of infection, however, the 52 k.Da fraction was detected in extracts from the brain, heart, lungs and kidneys of the infected mice. The 42 k.Da antigen was also detected in the kidneys of the infected mice. In infected rats both the 42 k.Da and 52 k.Da antigens were detected extravascularly in extracts of spleen and the 42 k.Da was also detectable kidneys extracts 17 days after infection, although both antigens were absent from the circulation. The variation in the fate of these antigens within the host body and the difference in their extravascular location in mice and rats suggests that the fate of individual trypanosome antigens is influenced by both the antigen itself and the host species. The difference in the dynamics and localisation of these trypanosome antigens warrants further investigation and provides a better understanding of the host-parasite interaction.

Antigens investigated in the present study were not detected in any cryostat sections examined. This might be due to their scarcity in these sections or due to changes taking place during the processing of the sections,

in particular the pre-fixation drying steps and the effect of acetone fixation on these particular antigenic molecules.

The rapid clearance of all three *T. evansi* antigens from the circulation after successful chemotherapy makes them good candidates for developing serologically-based test for measuring the effectiveness of chemotherapy. The 52 k.Da also has the advantage over the other two antigens in that it is an invariant antigen that appears early during infection and could also be a candidate antigen for the diagnosis of infection, but only in areas where diagnosis is not complicated by the presence of other salivarian trypanosomes as the antigen is present in both *T.brucei* and *T.vivax*. Furthermore, the fact that the 42 k.Da and 52 k.da antigens could be detected in tissue extracts of some organs even after they had disappeared from the blood raises the possibility of diagnosis of trypanosome infection based on such extracts, possibly even collected post-mortem.

CHAPTER EIGHT

GENERAL DISCUSSION

Scientific endeavour over the past 100 years has yielded large amounts of information on many aspects of the parasitic trypanosomes including morphology, infectivity, behaviour of intact parasites and host/parasite interaction and has been the subject of extensive reviews (Hoare, 1972, Stephen, 1986, Urquhart and Holmes, 1987). Our knowledge on many aspects of host/parasite interaction and immunology in particular, is mainly gleaned from studies based only on whole parasite extracts . Such extracts consist of a complex mixture of molecules each potentially capable of interacting with the host in a particular way. A full understanding of the way in which trypanosomes interact with the host must take into account the nature and behaviour of individual parasite components and the way in which they interact with the host. Recent developments in biotechnology have led to the emergence of a number of sophisticated techniques such as SDS-PAGE and immunoblotting (Towbin *et al*, 1979, Burnette, 1981) that are capable of resolving individual parasite components from complex mixtures and assessing their interaction with host components.

The discriminatory power of SDS-PAGE (Laemmli, 1970) has been used extensively for the analysis of pathogenic organisms such as trypanosomes. When combined with the technique of immunoblotting they provide a powerful means for examining host/parasite interaction at the molecular level. This combination of technique have been used in trypanosomiasis research mainly for characterisation and identification of the variant surface glycoprotein of trypanosomes (Cardoso de Almeida and Turner, 1983, Gardiner *et al*, 1987), determination of the molecular nature of invariant antigens in *T. brucei rhodesiense* (Burgess and Jerrells, 1985) and studying antibody response to *T. evansi* antigens in experimentally infected

rabbits (Uche, 1989). In the present study a similar combination of molecular techniques was used to identify and antigenically characterise individual *T. evansi* components using sera from experimentally infected rabbit and hyperimmune serum to a range of the parasite soluble materials. Overall twenty seven components were found to be antigenic but the number was dependent on the way in which they were presented to the host. The majority of these components acted as antigens during 21 days of infection. The others, although not recognised as antigens during the infection were capable of eliciting an antibody response when used as soluble extract. From those recognised during infection two antigens of 42 k.Da and 52 k.Da identified as major components in the immune response to *T. evansi* were selected for further investigation into the dynamics of *T. evansi* antigens during the course of infection.

For studies on the dynamics of the individual parasite antigens during infection, an assay capable of sufficient sensitivity for detecting and tracing these antigens in the host is needed. Antigen-capture ELISA is capable of detecting antigens in host materials and have been used successfully to detect circulating trypanosome antigens (Rae and Luckins, 1984; Nantulya *et al*, 1989, Olaho-Mukani *et al*, 1989; Masake and Nantulya, 1991, El Amin *et al*, 1993, Olaho-Mukani *et al*, 1993, Sinyangwe and Munyama, 1993). The study of the interaction of the antigens at tissue and cellular level is facilitated by the development of immunohistochemical techniques. Attempts were made to exploit both types of assays in the present study as a tool for studying the dynamics of individual antigens of *T. evansi*.

A key element in these immunoassays is the availability of high specific antibody to detect the target antigen as the sensitivity of these assays is dependent on the specific activity and high avidity of the antibody (Tijssen,

1985). Two types of antibodies can be utilised in these assays to detect the target antigen polyclonal antibodies in hyperimmune serum raised to a single antigen, and monoclonal antibodies. The hyperimmune serum usually contain high proportion of high affinity antibodies with high ability to bind antigen (Harlow and Lane, 1988). such high affinity and high sensitivity of polyclonal antibodies makes them better reagents in immunoassays compared to monoclonal antibodies which often has low affinity (Tijssen, 1985). However, the successful production of polyclonal antibodies requires the availability of a highly purified antigen so that the antibody produced will be able to detect the target antigen in complex mixtures. The production of monoclonal antibodies do not require pure antigen and are highly selective reagents that can be used in the identification and isolation of individual antigens in complex mixtures (Liddell and Cryer, 1991) as they usually recognise a single antigenic determinant. Another advantage of monoclonal antibodies over the polyclonal antibodies is that large quantities of identical antibodies can be generated. The present study utilised both type of antibodies, polyclonal antibodies to the 42 k.Da and 52 k.Da *T. evansi* antigens and a monoclonal antibody recognising a 72 k.Da antigen of *T. evansi*. Characterisations of these antigens revealed the 42 k.Da antigen to be variant-specific antigen, while the 52 k.Da antigen was common to different variants and stocks of *T. evansi* and to *T. brucei* and *T. vivax*. The 72 k.Da was stock specific antigen common between different variants of the same *T. evansi* stock.

In developing immunoassay for the detection of antigens purified antibodies are usually required as the efficiency of these assays is directly dependent on the purity of the antibody (Tijssen, 1985). Immunoglobulins constitute about 10% of the total serum proteins of which 1 - 10% are usually antibodies to the injected antigen (Kurstak, 1985). Enrichment of antibodies is

a necessary step achieved by purification and consequently the amount of enzyme needed to conjugate the antibody will be reduced. A number of purification methods are available of which affinity chromatography with protein A Sepharose is the most popular since it produces high yields of pure antibody (Tijssen, 1985, Harlow and Lane, 1988). Antibodies produced in the present study were purified using this method and the effect of purification on their detectability was tested by antibody-ELISA. The purified antibodies were then labelled with horseradish peroxidase and biotin before used to develop the antigen-capture ELISA.

The performance of any immunoassay depends largely on optimisation of the assay conditions (Kemeny, 1992). In order to optimise the conditions of the antigen-capture assay and to test its capability of detecting the target antigens in the present study the assay was first tested using crude *T. evansi* extracts. After establishing appropriate working conditions the assay was then evaluated against antigens in serum of a rabbit inoculated with a crude parasite extract and tissue extracts from infected mice. Cryostat sections were also prepared from these tissues and examined for the presence of the antigens. Extracts from infected and perfused organs were used as a second approach to tissue sections to determine the presence of the antigens in the extravascular sites. The assay was then applied for studying the dynamics of the antigens during the course of infection and after drug treatment in experimentally infected rats.

The assays developed were sufficiently stable and sensitive in that they were capable of detecting the target antigens in the blood and tissue. Differences were seen in the dynamics of the three antigens investigated in the present study. The 52 k.Da antigen reached a detectable level in the circulation of the infected rats before the appearance of the other two antigens, while the

72 k.Da antigen was the last to be detected. Following treatment of the infected rats the 72 k.Da antigen was the first to disappear from the circulation followed by the 42 k.Da and the 52 k.Da antigens two days later.

The antigen-capture ELISA used in the present study was also able to detect the target antigens in the tissue extracts collected from infected mice and rats. As in the circulation differences were seen in the location of the three antigens in these extravascular sites. The 42 k.Da antigen was detected 4 days after infection from the brain and spleen of the infected untreated mice possibly due to the presence of trypanosomes in these organs. Extravascular trypanosomes in the brain and spleen of infected animals have been reported (Seed and Effron, 1973, Turner *et al*, 1986, Sudarto *et al*, 1990) and considered as important sources of relapses after drug treatment. The 52 and the 72 k.Da antigens were however not detected in these tissues at this time of infection possibly due to their scarcity as they represent small proportion of the parasite extract. Following treatment the 42 k.Da antigen was detectable from the kidneys while the 52 k.Da was present in extracts from brain, heart, lungs and kidneys indicating differences in the behaviour of these antigens. In rats the antigens were detected in the tissues from 6 days following their disappearance from the circulation. The 42 k.Da antigen was detected from the kidney and spleen and the 52 k.Da antigen from the spleen. The 72 k.Da antigen was, however not detected from any of the tissues examined. In both infected mice and rats the clearance of the antigens appeared to be through the spleen and the kidneys as indicated by the presence of the antigens in these organs following their disappearance from the circulation.

The three antigens were not detected in the cryostat sections examined, possibly due to alteration encountered during the processing of the sections particularly the effect of the fixative used (acetone) (Tijssen, 1985).

The detection of the parasite antigens in extracts of tissues from perfused infected animals by the antigen-capture ELISA used in the present study represents a useful application of this sensitive test to study extravascular trypanosomes. The rapid clearance of the antigens from the circulation following the destruction of the trypanosomes by chemotherapy is a possible indicator of effective chemotherapy since their absence is indicative of the disappearance of the parasites from the circulation. This contrasts with other findings where antigens persisting in the circulation for periods of two months after the destruction of the parasites by chemotherapy have been reported in cattle (Sinyangwe and Munyama, 1993).

The release of internal antigens in the circulation depends on the destruction of the trypanosomes from the preceding parasitaemic wave and usually do not reach detectable levels before the remission of the first parasitaemia (Nantulya *et al*, 1986). The 52 k.Da antigen being a common trypanosome antigen that can reach a detectable level in the circulation before the remission of the first parasitaemic wave represent a possible candidate for the diagnosis of infection particularly in areas where *T. evansi* is the only trypanosome species present.

The differences in the behaviour and the fate of these antigens during infection warrant further investigation in the dynamics of the other antigenic components of the parasite using this approach. Also application of this approach for the parasite components in the primary hosts might require the use of more advanced techniques such as 2-dimensional electrophoresis which is capable of high resolution and can identify minor differences between the parasite components.

REFERENCES

Adams, A.R.D. and Lionnet, F.E. (1933). An outbreak of Surra among wild deer (*Cervus unicolor var.*) of Mauritius. *Journal of Comparative Pathology and Therapeutics*, **46:** 165-167.

Alves, M.J.M., Allison, A.C., and Nussenzweig, V. (1983). Monoclonal antibodies to *Trypanosoma cruzi* inhibit motility and nucleic acid synthesis of culture forms. *Infection and Immunity*, **39**:377-382.

Andrews, A.T. (1988). Electrophoresis: Theory, Techniques and Biochemical and Clinical Application. 2. Oxford. Clarendon Press.

Aubertin, A.M., Tondre. L., Lopez, C., Obert, G. and Kirn, A. (1983). Sodium dodecyl sulfate-mediated transfer of electrophoretically separated DNA-binding proteins. *Analytical Biochemistry*, **131:** 127-134.

Authie, E. (1993). Antibody responses to invariant antigens of *Trypanosoma congolense* in cattle of different susceptibility to trypanosomiasis. *Parasite Immunology*, **15**:101-111.

Avrameas, S. and Ternynck, T. (1971). Peroxidase labelled antibody and Fab conjugates with enhanced intracellular penetration. *Immunochemistry*, **8:** 1175.

Baker, J.R. (1970). Techniques for the detection of trypanosome infections. In: *The African Trypanosome* (ed. H.W. Mulligan). George Allen and Unwin, London. p. 67-88.

Barriga, O.O. (1981). The immunology of parasitic infections. A handbook for physicians, veterinarians and biologists. Baltimore. University Park Press.

Barry, J.D. and Vickerman, K. (1979). *Trypanosoma brucei*: loss of variable antigens during transformation from bloodstream to procyclic forms in vitro. *Experimental Parasitology*, **49**:281-300.

Bellanti, J.A. (1985). Immunology III. Philadelphia. W.B. Saunders Company.

Boreham, P.F.L. and Kimber, C.D. (1970). Immune complexes in trypanosomiasis of rabbit. *Transaction of The Royal Society of Tropical Medicine and Hygiene* , **64:** 168-169.

Boulard, C. and Lecroisey, A. (1982). Specific antisera produced by direct immunisation with slices of polyacrylamide gel containing small amounts of protein. *Journal of Immunological Methods*, **50**:221-226.

Bringaud, F. and Baltz, T. (1992). A potential hexose transporter gene expressed predominantly in the bloodstream form of *Trypanosoma brucei*. *Molecular and Biochemical Parasitology*, **52:** 111-122.

Brown, K.N. and Williamson, J. (1962). Antigen of *Brucei* trypanosomes. *Nature (London)*,**194:**1253-1255.

Burgess, D.E. and Jerrells, T. (1985). Molecular identity and location of invariant antigens on *Trypanosoma brucei rhodesiense* defined by monoclonal antibodies reactive with sera from trypanosomiasis patients. *Infection and Immunity*, **50**:893-899.

Burnette, W.N. (1981). "Western blotting". Electrophoretic transfer of proteins from sodium dodecyl sulphate-polyacrylamide gel to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Analytical Biochemistry*, **112**:195-203.

Campbell, G.H. and Phillips, S.M. (1976). Adoptive transfer of variant-specific resistance to *Trypanosoma rhodesiense* with B lymphocyte and serum.. *Infection and Immunity*, **14**:1144-1150.

Campbell, G.H., Esser, K.M. and Phillips, S.M. (1978). *Trypanosoma rhodesiense* infection in congenitally athymic (nude) mice. *Infection and Immunity*, **20:**714-720.

Capbern, A., Giroud, C., Baltz, T., and Mattern, P. (1977). *Trypanosoma equiperdum*: etude des variations antigenques au cours de la trypanosome experimentale du lapin. *Experimental Parasitology*, **42**:6-13.

Cardoso de Almeida, M.L. and Turner, M.J. (1983). The membrane form of variant surface glycoproteins of *Trypanosoma brucei*. *Nature*, **302**:349-352.

Carrol, R.B., Goldfine, S.M. and Mehero, J.A. (1978). Antiserum to polyacrylamide gel-purified simian virus 40 T antigen. *Virology*, **87:** 194-198.

Choudhury, A. and Misra, K.K. (1972). Experimental infection of *Trypanosoma evansi* in a carnivore host. *Transaction of The Royal Society of Tropical Medicine and Hygiene*, **66**:365-366.

Clarke, M.W., Olafson, R.W. and Pearson, T.W. (1984). Purification of major variable-surface glycoproteins from African trypanosomes by reverse-phase high-performance liquid chromatography. *Analytical Biochemistry*, **142:** 360-368.

Colmerauer, M.E.M., Davis, C.E. and Pearson, T.W. (1989). The trypanosome surface glycoprotein procyclin is expressed only on tsetse fly vector stages of the parasite. *Parasitology Research*, **76:** 171-173.

Coppens, I., Baudhuin, P., Opperdoes, F.R., and Courtoy, P.J. (1988). Receptors for the host low density lipoproteins on the hemoflagellate *Trypanosoma brucei*: Purification and involvement in the growth of the parasite. *Proceedings of the National Academy of Science, USA*, **85**:6753-6757.

Coppens, I., Bastin, PH., Opperdoes, F.R., Baudhuin, P. and Courtoy, P.J. (1992). Trypanosoma brucei brucei: antigenic stability of its LDL receptor and immunological cross-reactivity with LDL receptor of the mammalian host. *Experimental Parasitology*, **74:** 77-86.

Cross, G.A.M. (1977). Isolation, structure and function of variant-specific surface antigens. *Annales de la Societe Belge de Medecine Tropicale*, **57**:389-399.

Cross, G.A.M. (1979). Biochemistry of variant antigens. In: Pathogenicity of Trypanosomes. (ed. Losos, G. and Chouinard, A.), pp. 32-37. Ottawa. International Development Research Centre.

Cross, G.A.M. (1975). Identification, purification and properties of clone-specific glycoprotein antigens constituting the surface coat of *Trypanosoma brucei*. *Parasitology*, **71**:393-417.

Cross, G.A.M. (1990). Cellular and genetic aspects of antigenic variation in trypanosomes. *Annual Review of Immunology*, **8**:83-110.

Cross, G.A.M. (1990b). Glycolipid anchoring of plasma membrane proteins. *Annual Review of Cell Biology*, **6:** 1-39.

Cross, G.A.M. and Johnson, J.G. (1976). Structure and organisation of the variant specific surface antigens of *Trypanosoma brucei*. In: Biochemistry of Parasites and Host-Parasite Relationship. (ed. Van den Bossche, H.), pp. 413-420. Amsterdam. North Holland Publishing Company.

Cunningham, M.P. and Vickerman, K. (1962). Antigenic analysis in the *Trypanosoma brucei* group using the agglutination reaction. *Transaction of The Royal Society of Tropical Medicine and Hygiene*, **56**:48-59.

Curasson, G. (1943). Traite de protozoologie veterinaire et comparee. I. Trypanosomes, Paris.

De Gee, A.L.W. and Rovis, L. (1981). *Trypanosoma vivax*: absence of host proteins on the surface coat. *Experimental Parasitology*, **51**:124-132.

De Raadt, P. (1974). Immunity and antigenic variation: clinical observations suggestive of immune phenomena in African trpanosomiasis. In Trypanosomiasis and Leishmaniasis with Special Reference to Chagas› Disease. CIBA Foundation Symposium 20: (ed K. Elliott, M. O›Connor and G.E.W. Wolstenholme) p. 199-224. Associated Scientific Publisher, Amsterdam.

Desowitz, R.S. (1956). Effect of antibody on the respiratory rate of *Trypanosoma vivax*. *Nature (London)*,**177:**132-133.

Dignam, J.D. (1990). Preparation of extracts from higher Eukaryotes. In: *Methods in Enzymology: Guide to protein purification.* (ed. Deutscher, M.P.), Volume, **182:** 194-203. Academic Press Inc.

Donelson, J.E. and Turner, M.J. (1985). How the trypanosome changes its coat. *Scientific American*, **252(2):**32-39.

Down, J.A., Garman, S.C., Gurnett, A.M., Turner, M.J. and Wiley, D.C. (1991). Crystallization and preliminary X-ray analysis of an intact soluble-form variant surface glycoprotein from the African trypanosomes, *Trypanosoma brucei*. *Journal of Molecular Biology*, **218(4):**679-683.

Doyle, J.J. (1977). Antigenic variation in the salivarian trypanosomes. In: Immunity to Blood Parasites of Animals and Man. (ed. Miller, L.H., Pino, J.A, and McKelvey, J.J.), pp. 31-63. London. Plenum Press.

Doyle, J.J., Hirumi, H., Hirumi, K., Lupton, E.N. and Cross, G.A.M. (1980). Antigenic variation in clones of animal-infective *Trypanosoma brucei* derived and maintained in vitro. *Parasitology*, **80:**359-369.

Dresser, D.W. (1962). Specific inhibition of antibody production. II. Paralysis induced in adult mice by small quantities of antigen. *Immunology* **5:** 378-388.

Ekejindu, G.O.C., Maikaje, D.B. and Ekejindu, I.M. (1986). The combined procedures of Ficoll-Paque centrifugation and anion exchange separation for the recovery of trypanosomes from blood and other tissues. *Transaction of the Royal Society of Tropical Medicine and Hygiene*, **80:** 879-882.

El Amin, E.A., Hassan, E.M., El Bashir, M.O.A., and Luckins, A.G. (1993). Prevalence of trypanosomiasis in pastoral camels in the Butana plains, Mid-Eastern Sudan. In: Improving the diagnosis and control of trypanosomiasis and other vector-borne diseases of African livestock using immunoassay methods. *International Atomic Energy Agency (IAEA) Report*, 111-119.

Evans, G. (1881 - 1882). On a horse disease in India known as Surra, probably due to Haematozoan. Veterinary Journal, 13: 1-10, 82-88, 180-200, 326-332; 14: 97-110, 181-187.

Ferguson, M.A.J. (1994). What can GPI do for you?. *Parasitology Today*, **10 (2):**48-52.

Ferguson, M.A.J., Homans, S.W. (1989). The membrane attachment of the variant surface glycoprotein coat of *Trypanosoma brucei*. New Strategies in Parasitology (ed. K.P.W.J., McAdam). Proceedings of an international Symposium sponsored by Glaxo research, Brocket Hall, Hertfordshire, 22 - 25 April, 1989. Chuchill Livingstone, Edinburgh.

Ferguson, M.A.J., Homans, S.W., Dwek, R.A., and Rademacher, T.W. (1988). Glycosyl-phosphatidylinositol moiety that anchors *Trypanosoma brucei* variant surface glycoprotein to the membrane. *Science*, **239**:753-759.

Ferrante, A. and Allison, A.C. (1983). Alternative pathway activation of complement by African trypanosomes lacking a glycoprotein coat. *Parasite Immunology*, **5**:491-498.

Frommel, T.O. and Balber, A.E. (1987). *Trypanosoma brucei brucei*, *T. brucei gambiense* and *T. brucei rhodesiense*: Common glycoproteins and glycoprotein oligosaccharide heterogeneity identified by lectin affinity blotting and endoglycosidase H treatment. *Experimental Parasitology*, **63**:32-41.

Frommel, T.O., Seyfang, A., and Balber, A. (1988). *Trypanosoma brucei* sspp.: Cleavage of variant specific and common glycoproteins during exposure of live cells to trypsin. *Experimental Parasitology*, **66**:213-224.

Fruit, J., Afchain, D., Petitprez, A., Van Meirvenne, N., Le Ray, D., Bout, D., and Capron, A. (1977). Antigenic analysis of a variant-specific component of *Trypanosoma brucei*: localization on the surface coat with labelled specific antibodies. *Parasitology*, **74**:185-190.

Galfre, G. and Milstein, C. (1981). Preparation of monoclonal antibodies: strategies and procedures. *Methods in Enzymology*, **73:**3-46.

Gallo, J.M. and Schrevel, J. (1985). Homologies between paraflagellar rod proteins from trypanosomes and euglenoids revealed by monoclonal antibody. *European Journal of Cell Biology*, **36:** 163-168.

Galuzo, I.G. and Novinskaja, V.F. (1960). Trypanosomes of animals in Kazakhstan. Trudy Institute zoologii. *Akademiya Nauk Kazakh SSR*, **14:**3-11.

Gardiner, P.R., Pearson, T.W., Clarke, M.W., and Mutharia. (1987). Identification and isolation of a variant surface glycoprotein from *Trypanosoma vivax*. *Science*, **235:** 774-777.

Goding, J.W. (1980). Antibody production by hybridomas. *Journal of Immunological Methods*, **39**:285-308.

Goding , J.W. (1986). Monoclonal antibodies: principles and practice. Academic Press Inc. Ltd. London.

Gray, A.R. (1965). Antigenic variation in a strain of *Trypanosoma brucei* transmitted by *Glossina morsitans* and *G. palpalis*. *Journal of General Microbiology*, **41**:195-214.

Hall, T. and Esser, K. (1984). Topologic mapping of protective and non-protective epitopes on the variant surface glycoproteins of the WRAT at 1 clone of *Trypanosoma brucei rhodesiense*. *Journal of Immunology*, **132:** 2059-2063.

Harlow, E. and Lane, D. (1988). Antibodies a laboratory manual. Cold Spring Harbor Laboratory.

Henry, M.P. (1989). Ion-exchange chromatography of proteins and peptides. In: *HPLC of macromolecules - a practical approach.* (ed. Oliver, R.W.A..), pp. 91-126. Oxford. IRL Press.

Hide, G., Gray, A., Harrison, C.M., and Tait, A. (1989). Identification of an epidermal growth factor receptor homologue in trypanosomes. *Molecular and Biochemical Parasitology*, **36**:51-59.

Hoare, C.A. (1965). Vampire bats as vectors and hosts of equine and bovine trypanosomes. *Acta Tropica*, **22:** 204-209.

Hoare, C.A. (1972). The Trypanosomes of Mammals (A zoological monograph). Oxford. Blackwell Scientific Publications.

Hoeijmakers, J.H.J., Frash, A.C.C., Bernards, A., Brost, P., and Cross, G.A.M. (1980). Novel expression-linked copies of the genes for variant surface antigens in trypanosomes. *Nature (London)*,**284:**78-80.

Holmes, P.H., MacAskill, J.A., Whitelaw, D.D., Jennings, F.W., and Urquhart, G.M. (1979). Immunological clearance of ^{75}Se-labelled *Trypanosoma brucei* in mice. I. Aspects of the radiolabelling technique. *Immunology*, **36**:415-420.

Hughes, D.E. (1951). A press for disrupting bacteria and other organisms. *British Journal of Experimental Pathology*, **32**:97-109.

Iwamasa, T., Ninomiya, N., Hamada, T. and Fukuda, S. (1982). Immunohistochemical localisation of acid α-glucosidase in rat liver. A light and electron microscopic study. *The Journal of Histochemistry and Cytochemistry*, **30(4):** 378-384.

Jennings, F.W., Whitelaw, D.D., Holmes, P.H., Chizyuka, H.G.B., and Urquhart, G.M. (1979). The brain as a source of relapsing Trypanosoma brucei infection in mice after chemotherapy. *International Journal for Parasitology*, **9:**381-384.

Johnson, J.G. and Cross, G.A.M. (1977). Carbohydrate composition of varaint-specific surface antigen glycoprotein from *Trypanosoma brucei*. *Journal of Protozoology*, **24:**587-591.

Johnson, P., Neal, R.A., and Gall, D. (1963). Protective effect of killed trypanosome vaccines with incorporated adjuvants. *Nature (London)*, **200:**83.

Johnstone, A. and Thorpe, R. (1982). Immunochemistry in Practice. Oxford. Blackwell Scientific Publication.

Kemeny, D.M. (1992). Titration of antibodies. *Journal of Immunological Methods*, 150: 57-76.

kohler, G. and Milstein, C. (1975). Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature*, **256:**495-497.

Kurstak, E. (1985). Progress in enzyme immunoassays: production of reagents, experimental design, and interpretation. *Bulletin of the World health Organisation*, **63(4):** 793-811.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227:**680-685.

Lanham, S.M. and Godfrey, D.G. (1970). Isolation of salivarian trypanosomes from man and other mammals using DEAE cellulose. *Experimental Parasitology*, **28:** 521-534.

Lazarides, E. and Weber, K. (1974). Actin antibody: The specific visualization of actin filaments in non-muscle cells. *Proceedings of the National Academy of Science* USA, **17:**2268-2272.

Leppi, T.J., Repesh, L.A., Furcht, L.T., Bartzen, P.J. and Holt, G.E. (1982). Immunohistochemical localisation of fibronectin in human and rat uterine cervices. *The journal of Histochemistry and Cytochemistry*, **30(5):** 413-417.

Liddell, J.E and Cryer, A. (1991). A Practical Guide to Monoclonal antibodies. Chichester. John Wiley & Sons Ltd..

Lis, H. and Sharon, N. (1973). The biochemistry of plant lectins (phytohemagglutinins). *Annual Review of Biochemistry*, **42:** 541-574.

Lonsdale-Eccles, J.D. and Mpimbaza, G.W.N. (1986). Thiol dependent proteins of African trypanosomes: analysis by electrophoresis in sodium dodecyl sulphate polyacrylamide gels co-polymerised with fibrinogen. *European Journal of Biochemistry*, **155:** 469-473.

Lonsdale-Eccles, J.D. and Grant, D.J. (1987). Purification of African trypanosomes can cause biochemical changes in the parasite. *Journal of Protozoology*, **34:** 405-408.

Losos, G.J. and Ikede, B.O. (1972). Review of the pathology of diseases in domestic and laboratory animals caused by *Trypanosoma congolense*, *T. vivax*, *T. brucei*, *T. rhodesiense* and *T. gambiense*. *Veterinary Pathology*, **9:** 1-71.

Luckins, A.G. (1988). *Trypanosoma evansi* in Asia. *Parasitology Today*, **4(5):**137-142.

Lumsden, W.H.R. and Herbert, W.J. (1967). Phagocytosis of trypanosomes by mouse peritoneal macrophages. *Transaction of The Royal Society of Tropical Medicine and Hygiene*, **61:**142.

Low, M.G. (1989). The glycosyl-phosphatidylinositol anchor of membrane proteins. *Biochemica et Biophysica Acta*, **988:** 427-454.

MacAskill, J.A., Holmes, P.H., Whitelaw, D.d., McConnell, I., Jennings, F.W., and Urquhart, G.M. (1980). Immunological clearance of ^{75}Se-labelled *Trypanosoma brucei* in mice. II. Mechanisms in immune animals. *Immunology*, **40:**629-635.

Marinkelle, C.J. (1976). The biology of the trypanosomes of non-human primates. In: Biology of the Kinetoplastida. (ed. Lumsden, W.H.R. and Evans, D.A.), pp. 232. Academic Press.

Masake, R.A. and Nantulya, V.M. (1991). Sensitivity of an antigen detection enzyme immunoassay for diagnosis of *Trypanosoma congolense* infections in goats and cattle. *Journal of Parasitology*, **77(2):** 231-236.

Masterson, W.J., Taylor, D., and Turner, M.J. (1988). Topologic analysis of the epitopes of a variant surface glycoprotein of Trypanosoma brucei. *Journal of Immunology Baltimore*, **140:**3194-3199.

Miller, E.N. and Turner, M.J. (1980). Analysis of antigenic types appearing in first relapse populations of clones of *Trypanosoma brucei*. *Parasitology*, **82:**63-80.

Mohammad, K. and Esen, Asim, (1989). A blocking agent and blocking step are not needed in ELISA, immunostaining dot-blots and Western blots. *Journal of Immunological Methods*, **117:** 141-145.

Morrison, W.I., Black, S.J., Paris, J., Hinson, C.A., and Wells, P.W. (1982). Protective immunity and specificity of antibody responses elicited in cattle with irradiated *Trypanosoma brucei*. *Parasite Immunology*, **4:**395-407.

Muller, N., Hemphill, A., Imboden, M., Duvallet, G., Dwinger, R.H., and Seebeck, T. (1992). Identification and characterisation of two repetitive non-variable antigens from African trypanosomes which are recognised early during infection. *Parasitology*, **104(1):**111-120.

Mulumba, T.M. and Wery, M. (1983). Experimental infection with stocks of *Trypanosoma brucei gambiense*: study of the evolution by elution techniques of tissues. *Contributions in Microbiology and Immunology*, **7:** 120-129.

Murray, M. and Urquhart, G.M. (1977). Immunoprophylaxis against African trypanosomiasis. In: Immunity to Blood Parasites of Animals and Man. (ed. Miller, L.H, Pino, J.A. and McKelvery, J.J.), pp.209. New York. Plenum Press.

Murta, A.C.M., Persechini, P.M., Souto-Padron, T., Souza, W., Guimaraes, J.A., Scharfstein, J., De-Souto-Padron, T., and De-Souza, W. (1990). Structural and functional identification of GP57/51 antigen of *Trypanosoma cruzi* as a cysteine proteinase. *Molecular and Biochemical Parasitology*, **43:**27-38.

Musoke, A.J., Nantulya, V.M., Barbet, A.F., Kironde, F., and McGuire, T.C. (1981). Bovine immune response to African trypanosomes: specific antibodies to variable surface glycoproteins of *Trypanosoma brucei*. *Parasite Immunology*, **3:**97-106.

Mwangi, D.M., Hopkins, J. and Luckins, A.G. (1990). Cellular phenotypes in *Trypanosoma congolense* infected sheep: the local skin reaction. *Parasite Immunology*, **12:** 647-658.

Myler, P.J., Allen, A.L., Agabian, N., and Stuart, K. (1985). Antigenic variation in clones of *Trypanosoma brucei* grown in immune deficient mice. *Infection and Immunity*, **47:**684-690.

Nagle, R.B., Ward, P.A., Lindsey, H.B., Sandun, E.H., Johnson, A.J., Berkawe, K.E., and Hilderbrandt, P.K. (1974). Experimental infections with African trypanosomes, VI. glomerulonephritis involving the alternate pathway of complement activation. *American Journal of Tropical Medicine and Hygiene*, **23:**15-26.

Nantulya, V.M. and Doyle, J.J. (1977). Stabilization and preservation of the antigenic specificity of *Trypanosoma* (*Trypanozoon*) *brucei* variant

specific surface antigens by mild fixation techniques. *Acta Tropica*, **34:**313-320.

Nantulya, V.M., Musoke, A.J., Barbet, A.F., and Roelants, G.E. (1979). Evidence for reappearance of *Trypanosoma brucei* variable antigen types in relapse populations. *Journal of Parasitology*, **65:**673-679.

Nantulya, V.M., Musoke, A.J., Ito, S., Minja, S.H. and Saigar, N. (1986). Identification of a species-specific *Trypanosoma vivax* antigen for use in diagnosis. *Parasitology-Quo Vadit, Proceedings of the VI International Congress of Parasitology*, Brisbane, Australia, (ed. M.J. Howell). Australian Academy of Science, Canberra. p. 196.

Nantulya, V.M. Musoke, A.J., Rurangirwa, F.R. Saigar, N. and Minja, S.H. (1987). Monoclonal antibodies that distinguish *Trypanosoma congolense*, *T. vivax* and *T. brucei*. *Parasite Immunology*, **9:** 421-431.

Nantulya, V.M., Bajyana-Songa, E., and Hamers, R. (1989). Detection of circulating trypanosomal antigens in *Trypanosoma evansi*-infected animals using a *T. brucei* group-specific monoclonal antibody. *Tropical Medicine and Parasitology*, **40:**263-266.

Nantulya, V.M. and Lindqvist, K.J. (1989). Antigen-detection enzyme immunoassays for the diagnosis of *Trypanosoma vivax*, *T. congolense* and *T. brucei* infections in cattle. *Tropical Medicine and Parasitology*, **40:**267-272.

Naritoku, W.Y. and Taylor, C.R. (1982). A comparative study of the use of monoclonal antibodies using three different immunohistochemical methods: An evaluation of monoclonal and polyclonal antibodies against human prostatic acid phosphate. *The Journal of Histochemistry and Cytochemistry*, **30(3):** 253-260.

Newton, B.A. (1981). African trypanosomiasis: Improved control by a genetic approach. In: Modern genetic concepts and techniques in the study of parasites. (ed. Michal, F.), pp.173-194. Basel. Schwabe & Co.

North, M.J., Coombs, G.M. and Barry, J.D. (1983). A comparative study of the proteolytic enzymes of *T. brucei*, *T. equiperdum*, *T. evansi*, *T. vivax*, *Leishmania tarentolae* and *Crithidia fasiculata*. *Molecular and Biochemical Parasitology*, **9:** 161-180.

Nossal, G.J.V. (1993). Life, death and the immune system. *Scientific American*, **269:**21-30.

Nygren, H. (1982). Conjugation of horseradish peroxidase to Fab fragments with different homobifunctional and heterobifunctional cross-linking reagents. *Journal of Histochemistry and Cytochemistry*, **30:**407-412.

O›Farrell, P.H. (1975). High resolution two-dimensional electrophoresis of proteins. *Journal of Biological Chemistry*, **250:** 4007-4021.

Oi, V.T and Herzenberg L.A. (1980). Immunoglobulin-producing hybrid cell lines. In: Selected Methods in Cellular Immunology. (ed. Mishell, B.B. and Shiigi, S.M.), pp. 351-372. San Fransisco, CA. Freeman.

Oi, V.T., Jones, P.P., Goding, J.W., and Herzenberg, L.A. (1978). Properties of monoclonal antibodies to mouse Ig allotypes, H-2 and Ia antigens. *Current Topics in Microbiology and Immunology*, **81:**115-129.

Olaho-Mukani, W., Munyua, W.K., Njogu, A.R., Mutugi, M.W., and Oketch, G. (1989). Diagnosis of *Trypanosoma brucei evansi* infection using a monoclonal sandwich ELISA technique. *KEMRI/KETRI Annual Medical Scientific Conference*, 112-114.

Olaho-Mukani, W., Munyua, W.K., Mutugi, M.W., and Njogu, A.R. (1993). Comparison of antibody- and antigen-detection enzyme immunoassays for the diagnosis of *Trypanosoma evansi* infections in camels. *Veterinary Parasitology*, **45:**231-240.

Ormerod, W.E and Venkatesan, S. (1971a). The occult visceral phase of mammalian trypanosomes with special reference to the life cycle of *Trypanosoma (Trypanozoon) brucei. Transaction of the Royal Society of Tropical Medicine and Hygiene*, **65:** 722-735.

Ormerod, W.E. and Venkatesan, S. (1971b). An amastigote phase of the sleeping sickness trypanosome. *Transaction of the Royal Society of Tropical Medicine and Hygiene*, **65:** 736-741.

Ormerod, W.E. and Raseroka, B.H. (1988). Screening compounds for sleeping sickness therapy without relapse. *Bulletin de la Societe de Pathologie Exotique et de ses Filiales*, **81:**543-547.

Overath, P., Chaudhri, M., Steverding, D., and Ziegelbauer, K. (1994). Invariant surface proteins in bloodstream forms of *Trypanosoma brucei. Parasitology Today*, **10(2):**53-58.

Parsons, M.N. and Nielsen, B. (1990). Active transport of 2-deoxy-D-glucose in *Trypanosoma brucei* procyclic forms. *Molecular and Biochemical Parasitology*, **42:**197-204.

Paulnock, D.M, Smith, C., and Mansfield, J.M. (1988). Antigen presenting cell function in African trypanosomiasis. Antigen presenting cells. Diversity, differentiation and regulation. Proceeding of a symposium held in Richmond, Virginia, March 26-29, 1987. (ed. Schook, L.B. and Tew, J.G.I.), pp.135-143. New York. Alan R. Liss, Inc..

Payne, R.C. (1988). Studies on the epidemiology of *Trypanosoma evansi* in the republic of Indonesia, M.Phil Thesis, University of Edinburgh.

Pearson, T.W. and Anderson, N.L. (1980). Analytical techniques for cell fractions. XXVIII. Dissection of complex antigenic mixtures using monoclonal antibodies and two-dimensional gel electrophoresis. *Analytical Biochemistry*, **101:**377-386.

Pearson, T.W., Pinder, M., Roelants, G.E., Kar, S.H., Lundin, L.B., Mayor-Withey, K.S., and Hewett, R.S. (1980). Methods for derivation and detection of antiparasite monoclonal antibodies. *Journal of Immunological Methods*, **34:**141-154.

Porstmann, B., Porstmann, T.T., Nugel, E., Evers, U. (1985). Which of the commonly used marker enzymes gives the best results in colorimetric and fluorimetric enzyme immunoassays: horseradish peroxidase, alkaline phosphatase, β-galactosidase?. *Journal of Immunological Methods*, **79:** 27-37.

Rae, P.F. and Luckins, A.G. (1984). Detection of circulating antigens by enzyme immunoassay. *Annals of Tropical Medicine and Parasitology*, **78:**587-596.

Rautenberg, P., Reinwald, E. and Risse, H.J. (1980). Evidence for Concanavalin A binding sites on the surface coat of *Trypanosoma congolense*. *Parasitology*, **80:** 113-122.

Reinwald, E., Risse, H.J., and Salker, R. (1978). Diazoniobenzene-sulphonate as a marker for cell surface proteins: study of the surface coat of *Trypanosoma congolense*. *Hope-Seylers Zeitschrift fur Physiologische Chemie*, **359:**939-944.

Reinwald, E., Rautenberg, P. and Risse, H.J. (1979). *Trypanosoma congolense*: mechanical removal of the surface coat in vitro. *Experimental Parasitology*, **48:**384-397.

Reynolds, E.S. (1963). The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *Journal of Cell Biology*, **17:** 208.

Reynolds, J.A. and Tanford, C. (1970). Binding of dodecyl sulfate to proteins at high binding ratios. Possible implications for the state of proteins in biologicla membranes. *Proceedings of the National Academy of Science, USA*, **66:**1002-1007.

Richards, F.F. (1984). The surface of the African trypanosomiasis. *Journal of Protozoology*, **31(1):**60-64.

Richards, F.F., Rosen, N.L., Onodera, M., Bogucki, M.S., Neve, R.L., Hotez, P., Armstrong, M.Y.K., and Konigsberg, W.H. (1981). Antigenic variation and the surface glycoproteins of *Trypanosoma congolense*. *Federation Proceedings of the American Societies for Experimental Biology and Medicine*, **40:**1434-1439.

Ritz, H. (1916).Uber Rezidive bei experimenteller Trypanosomiasis. II. Mitteilung. *Archiv fur Schiffs und Tropenhygiene*, **20:**397-420.

Roelants, G.E. and Pinder, M. (1984). Immunobiology of African trypanosomiasis. *Contemporary Topics in Immunobiology*, **12:**255-274.

Rolin, S., Halleux, S., Van Sande, J., Dumont, J., Pays, E., and Steinert, M. (1990). Stage-specific adenylate cyclase activity in *Trypanosoma brucei*. *Experimental Parasitology*, **71:** 350-352.

Ross, C.A., Cardoso de Almeida, M.C., and Turner, M.J. (1987). Variant surface glycoproteins of *Trypanosoma congolense* bloodstream and metacyclic forms are anchored by a glycolipid tail. *Molecular and Biochemical Parasitology*, **22:**153-158.

Rovis, L., Barbet, A.F., and Williams, R.O. (1978). Characterisation of the surface coat of *Trypanosoma congolense*. *Nature (London)*, **271:**654-656.

Schmidt, H. and Bafort, J.M. (1987). African trypanosomiasis: Haematogenic brain parasitism early in experimental infection through bypassing the blood-brain barrier, with considerations on brain trypanosomiasis in man. *Parasitology Research*, **73:** 15-21.

Seed, J.R. (1977). The role of immunoglobulins in immunity to *Trypanosoma brucei gambiense*. *International Journal of Parasitology*, **7**:55-60.

Seed, J.R., Cornille, R.L., Risby, E.L., and Gam, A.A. (1969). The presence of agglutinating antibody in the IgM immunoglobulin fraction of rabbit antiserum during experimental African trypanosomiasis. *Parasitology*, **59**:283-292.

Seed, J.R., Edwards, R., and Sechelski, J. (1984). The ecology of antigenic variation. *Journal of Protozoology*, **31(1)**:48-53.

Seed, J.R. and Effron, H.G. (1973). Simultaneous presence of different antigenic populations of *Trypanosoma brucei gambiense* in *Microtus montanus*. *Parasitology*, **66**:269-278.

Seed, J.R. (1972). *Trypanosoma gambiense* and *T. equiperdum*: Characterisation of variant specific antigens. *Experimental Parasitology*, **31**:98-108.

Shakibaei, M. and Frevert, U. (1992). Cell surface interactions between *Trypanosoma congolense* and macrophages during phagocytosis in vitro. *Journal of Protozoology*, **39(1)**:224-235.

Shapiro, S.Z. and Murray, M. (1982). African trypanosome antigens recognised during the course of infection in N›dama and Zebu cattle. *Infection and Immunity*, **35(2)**:410-416.

Sinha, P.K., Mikherjee, G.S., and Lahiri, R.K. (1971). Outbreak of *Trypanosoma evansi* amongst tigers and jaguars in the zoological gardens, Calcutta. *Indian Veterinary Journal*, **48**:306-310.

Sinyangwe, L.N. and Munyama, G.M. (1993). Field validation and use of the antigen-ELISA to monitor the effectiveness of a tsetse and trypanosomiasis control campaign in the Gwembe valley, Zambia. In: Improving the diagnosis and control of trypanosomiasis and other vector-borne diseases of African livestock using immunoassay methods. *International Atomic Energy Agency (IAEA) Report*, 87-93.

Stanworth, D.R. and Turner, M.W. (1978). Immunochemical analysis of immunoglobulins and their subunits. In: *Handbook of experimental immunology, volume* 1: *Immunochemistry*. (ed. Weir, D.M.), pp.241-253. Oxford. Blackwell Scientific Publications.

Stephen, L.E. (1986). Trypanosomiasis a veterinary prospective. Oxford. Pergamon Press.

Stieger, J. and Seebeck, T. (1986). Monoclonal antibodies against a 60 k.Da phenothiazine-binding protein from *Trypanosoma brucei* can discriminate between different trypanosome species. *Molecular and biochemical Parasitology*, **21:** 37-45.

Strauss, A.W., Alberts, A.W., Hennessy, S. and Vagelos, P.R. (1975). Regulation of synthesis of hepatic fatty acid synthetase: Polysomal translation in a cell-free system. *Proceedings of the National Academy of Science*, USA, **72:** 4366-4370.

Strickler, J.E., Mancini, P.E., and Patton, C.L. (1978). *Trypanosoma brucei brucei*: isolation of the major surface coat glycoprotein by lectin affinity chromatography. *Experimental Parasitology*, **46:**262-276.

Stryer, L. (1988). Biochemistry. 3rd edition. New York. W.H. Freeman and Company.

Stumph, W.E., Elgin, S.C.R. and Hood, L.E. (1974). Antibodies to protein dissolved in sodium dodecyle sulfate. *Journal of Immunology*, **113:**1752-1756.

Sudarto, M.W., Tabel, H., and Haines, D.M. (1990). Immunohistochemical demonstration of *Trypanosoma evansi* in tissues of experimentally infected rats and a naturally infected water buffalo (*Bubalus bubalis*).. *Journal of Parasitology*, **76(2):**162-167.

Takayanagi, T. and Enriquez, G.L. (1973). Effect of IgG and IgM immunoglobulins in *Trypanosoma gambiense* infections in mice. *Journal of Parasitology*, **59:**644-647.

Takayanagi, T., Kawaguchi, H., Yabu, Y., Itoh, M., and Appawu, M.A. (1987). Contribution of the complement system to antibody-mediated binding of *Trypanosoma gambiense* to macrophages. *Journal of Parasitology*, **73(2):**333-341.

Takayanagi, T., Kawaguchi, H., Yabu, Y., Itoh, M., and Yano, K. (1991). Dissociation of IgG antibody-mediated clumps of *Trypanosoma brucei gambiense* by complement. *Parasitology Research*, **77(8):**645-650.

Takayanagi, T., Kawaguchi, H., Yabu, Y., Itoh, M., and Yano, K. (1991). Immune mechanism facilitating clearance of *Trypanosoma gambiense*

by IgG3 antibody from infected host. *Tropical Medicine and Parasitology*, **42(4):**394-398.

Takayanagi, T., Kawaguchi, H., Yabu, Y., Itoh, M., and Yano, K. (1992). Diversity of complement mediated immune reaction to *Trypanosoma gambiense*. *Southeast Asian Journal of Tropical Medicine and public Health*, **23(1):**87-91.

Takayanagi, T., Kawaguchi, H., Yabu, Y., Itoh, M., and Yano, K. (1992). Inhibition of IgM antibody-mediated aggregation of *Trypanosoma gambiense* in the presence of complement. *Experientia*, **48(10):**1002-1006.

Takayanagi, T. and Nakatake, Y. (1975). *Trypanosoma gambiense*: enhancement of agglutinin and protection in sub-populations by immune spleen cells. *Experimental Parasitology*, **38:**233-239.

Tanner, M., Jenni, L., Hecker, H., and Brun, R. (1980). Characterisation of *Trypanosoma brucei* from lymph nodes of rats. *Parasitology*, **80:**383-391.

Taylor, D.W. and Cross, G.A.M. (1977). The synthesis of variant-specific antigen of *Trypanosoma brucei* in vitro. *Parasitology*, **74:**47-60.

Theodos, C.M., Reinitz, D.M., and Mansfield, J.M. (1990). Regulation of B cell responses to the variant surface glycoprotein (VSG) molecule in trypanosomiasis. I. Epitope specificity and idiotypic profile of monoclonal antibodies to the VSG of Trypanosoma brucei rhodesiense. *Journal of Immunology Baltimore*, **144:**4011-4021.

Tijssen, P. (1985). Practice and Theory of Enzyme Immunoassay. Vol. 15 of Laboratory Techniques in Biochemistry and Molecular Biology. 1. Amsterdam. Elsevier Science Publishers.

Tijssen, P. and Kurstak, E. (1983). An efficient two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis method for simultaneous peptide mapping of protein contained in a mixture. *Analytical Biochemistry*, **128:** 26-35.

Tizard, I. (1992). Macrophages and antigen processing. In: Veterinary Immunology. (ed. Tizard, I.), pp. 27-40. W.B. Saunders Company, Philadelphia.

Towbin, H., Staehelin, T., and Gordon, J. (1979). Electrophoretic transfer of proteins from ploy acrylamide gels to nitrocellulose sheets: procedure and some applications. *Proceedings of the National Academy of Science, USA*, **76:**4350-4354.

Tsang, V.C.W., George, E.B. and Hancock, K. (1985). Enzyme-linked immunoelectrotransfer blot (EITB). In: Enzyme-mediated immunoassays. (ed. Ngo, T.T. and Lenhoff, H.M.), Plenum Press, New York.

Turner, C.M.R., Hunter, C.A., Barry, J.D., and Vickerman, K. (1986). Similarity in variable antigen type composition of Trypanosoma brucei rhodesiense populations in different sites within the mouse host. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **80:**824-830.

Turner, M.J. (1982). Biochemistry of the variant surface glycoproteins of Salivarian trypanosomes. *Advances in Parasitology*, **21:**69-153.

Turner, C.M.R., Menrath, M. and Tait, A. (1989). Life-cycle-stage-and stock-specific labelling by monoclonal antibodies of *Trypanosoma brucei* procyclic forms. *Parasitology Research*, **75:** 414-415.

Uche, U.E. (1989). Immunological studies of *Trypanosoma evansi* infections in rabbits. *Ph. D. thesis,* University of Edinburgh.

Urquhart, G.M and Holmes, P.H. (1987). African Trypanosomiasis. Immune Responses in Parasitic Infections: Immunology, Immunopathology and Immunoprophylaxis. Volume III: Protozoa. pp.1-25. Boca Ratons, Florida. CRS Press Inc.

Van der Ploeg, L.H.T., Valerio, D., De Lange, T., Bernards, A., Brost, P., and Grosveld, F.G. (1982). An analysis of cosmid clones of nuclear DNA from *Trypanosoma brucei* shows that the genes for variant surface glycoproteins are clustered in the genome. *Nucleic Acids Research*, **10:** 5905-5923.

Vervoort, T., Barbet, A.F., Musoke, A.J., Magnus, E., Mpimbaza, G., and Van Meirvenne, N. (1981). Isotypic surface glycoproteins of trypanosomes. *Immunology*, **44:**223-232.

Vickerman, K. (1969). On the surface coat and flagellar adhesion in trypanosome. *Journal of Cell Science*, **5:**163-193.

Vickerman, K. and Luckins, A.G. (1969). Localisation of variable antigens in the surface coat of *Trypanosoma brucei* using ferritin conjugated antibody. *Nature (London)*, **224:**1125-1126.

Voller, A. and Bidwell, D.E. (1975). A simple method for detecting antibodies to *Rubella*. *British Journal of Experimental Pathology*, **56:** 338-339.

Voller, A., Bidwell, D.E. and Bartlett, A. (1979). The enzyme linked immunosorbent assay. A guide with abstracts of microplate applications (Dynatech Europe, Guernsey).

Webster, P. and Shapiro, S.Z. (1990). *Trypanosoma brucei*: A membrane-associated protein in coated endocytotic vesicles. *Experimental Parasitology*, **70:**154-163.

Webster, P., Russo, D.C.W., and Black, S.J. (1990). The interaction of *Trypanosoma brucei* with antibodies to variant surface glycoproteins. *Journal of Cell Science*, **96:**249-255.

Weitz, B. (1960). The properties of some antigens of *Trypanosoma brucei*. *Journal of General Microbiology*, **23:**589-600.

Wellde, B.T., Hockmeyer, W.T., Kovatch, R.M., Bhogal, M.S., and Diggs, C.L. (1981). *Trypanosoma congolense*: Natural and acquired resistance in the bovine. *Experimental Parasitology*, **52:**219-232.

Welling, G. and Welling-Wester, S. (1989). Size-exclusion HPLC of proteins. In: *HPLC of macromolecules - a practical approach.* (ed. Oliver, R.W.A.), pp.77-90. Oxford. IRL Press.

Wells, P.W., Emery, D.L., Hinson, C.A., Morrison, W.I., and Murray, M. (1982). Immunisation of cattle with variant specific surface antigen of *Trypanosoma brucei*: the influence of different adjuvants. *Infection and Immunity*, **36:**1-10.

WHO. (1976). The enzyme-linked immunosorbent assay (ELISA). *Bulletin of the World Health Organisation*, **54:** 129-139.

WHO. (1977). The role of immune complexes in diseases. *World Health Organisation Technical Report Series*, No. 606. 58 pp.

Wilson, M.B. and Nakane, P.K. (1978). In: Immunofluorescence and Related Techniques (eds.W. Knapp, H. Holubar and G. Wick). p. 215. Elsevier, Amsterdam.

Wright, K.A. and Hales, H. (1970). Cytochemistry of the pellicle of bloodstream forms of *Trypanosoma* (*Trypanozoon*) *brucei*. *Journal of Parasitology*, **56:**671-683.

Woo, P.T.K. (1970). The haematocrit centrifugation technique for the diagnosis of African trypanosomiasis. *Acta Tropica*, **27:**384-386.

Zahalsky, A.C. and Weinberg, R.L. (1976). Immunity to monomorphic *Trypanosoma brucei*: Humoral response. *Journal of Parasitology*, **62:**15-19.

Ziegelbauer, K., Multhaup, G., and Overath, P. (1992). Molecular Characterization of Two Invariant Surface Glycoproteins Specific for the Bloodstream Stage of *Trypanosoma brucei*. *The Journal of Biological Chemistry*, **267(15):**10797-10803.

Ziegelbauer, K. and Overath, P. (1992). Identification of Invariant Surface Glycoproteins in the Bloodstream Stage of *Trypanosoma brucei*. *The Journal of Biological Chemistry*, **267(15):**10791-10796.

APPENDICES

Appendix 1:
Phosphate buffered saline (PBS):

Sodium chloride	8.0 g.
Potassium chloride	0.2 g.
Disodium hydrogen phosphate, anhydrous	1.15 g.
Potassium dihydrogen phosphate	0.2 g.

Made up to one litre with distilled water.
pH 7.3

Appendix 2:
Phosphate saline glucose (PSG):

Disodium hydrogen phosphate, anhydrous	13.48 g.
Sodium dihydrogen phosphate	0.78 g.
Sodium chloride	4.25 g.

Made up to one litre with distilled water.

For use, the above solution was diluted in the ratio of 6:4 with distilled water and glucose was added to give a 1% solution. pH 8.0

Appendix 3:
Gel solutions:

solution required (in ml)	concentration of gel solution 20%	15%	10%	7% 4.5%
stock acrylamide (40%)	100	75	50	35	11.25
x4 lower tris	50	50	50	50	--
x4 upper tris	--	--	--	--	25
grams of sucrose	30	30	--	--	--
distilled water	20	45	100	115	63.75
Total volume	200*	200*	200*	200*	100**

* stored at -20°C in 20 ml aliquots.
** stored at -20°C in 10 ml aliquots.

Appendix 4:
Electrode and protein elution buffer:

Tris	3.03 g.
Glycine	14.41 g.
SDS	1.00 g.

Made up to one litre in distilled water.

Appendix 5:
Destain solution:

Methanol	25%.
Glacial acetic acid	10%.
Distilled water	65%.

Appendix 6:
Transfer buffer:

Electrode buffer (Appendix 4)	100 ml.
Methanol	100 ml.
Distilled water	300 ml.

Appendix 7:
Blocking buffer x10:

Tris	60.5 g (50 mM).
Sodium chloride	87.0 g (150 mM).
EDTA	3.72 g (1 mM).
Nonidet P40 (NP40)	5.0 ml (0.05%).
Gelatin	25.0 ml (0.25%).
Thiomersal	2.0 g (0.02%).

Distilled water to 1000 ml.
pH adjusted to 7.4 with concentrated HCl. For use the buffer was diluted 1:10 with distilled water and 5% Marvel added.

Appendix 8:
Substrate solution (immunoblot)**:**

(a) 60 mg 4-chloro-1-naphthol was dissolved in 20 ml ice-cold methanol.

(b) Tris buffered saline (TBS)

Tris	4.85 g (20 mM).
Sodium chloride	58.48 g (500 mM).

Made up to 2 litre with distilled water and pH adjusted to 7.5 with concentrated HCl.

(c) Immediately before use, 60 µl ice-cold hydrogen peroxide was added to 100 ml of TBS at room temperature, mixed with (a) and used in the assay.

Appendix 9:
ELISA coating buffer:(carbonate/bicarbonate buffer). pH 9.6

Sodium carbonate	1.59 g
Sodium bicarbonate	2.93 g

Made up to one litre with distilled water

Appendix 10:

ELISA washing buffer (PBS/Tween): pH 7.4

Sodium hydrogen orthophosphate	2.9 g
potassium dihydrogen orthophosphate	0.2 g
Sodium chloride	8.0 g
Potassium chloride	0.2 g
Tween 20	0.5 ml

Made up to one litre with distilled water.

Appendix 11:

Borate buffer: pH 8.0.

Borax (disodium tetraborate)	0.096 g (0.03 M)
Boric acid	0.140 g (0.23 M)
Sodium chloride	0.087 g (0.15 M)

Made up to 10 ml with distilled water. For use glucose was added to give a 1% solution.

Appendix 12:

SDS-sample buffer:

0.5M Tris-HCl, pH 6.8	1.0 ml.
10% (w/v) SDS	1.6 ml.
Dithiothreitol (DTT)	0.1 ml (123 mg).
Glycerol	0.8 ml.
Distilled water	4.4 ml.
0.05% (w/v) Bromophenol Blue	0.2 ml.

Appendix 13:

Sodium cacodylate buffer:

Sodium cacodylate 2.14 g

made up to 500 ml with distilled water. pH adjusted to 7.3 with HCl and stored at 4°C. Before use the buffer is double diluted with distilled water to make a single strength buffer. 12 ml of 25% EM grade gluteraldehyde is then added to 88 ml of the single strength (0.1 M) cacodylate buffer, pH 7.3.

Appendix 14:

Phosphate buffer:(0.015M)

Disodium hydrogen phosphate (Na_2HPO_4) 2.13 g

Made up to one litre with distilled water

pH adjusted to 8.0 with HCl

Appendix 15:

HPLC: Buffer A:

Tris 20 mM.

pH adjusted to 7.0 with HCl

Appendix 16:
HPLC: Buffer B:

Tris	20 mM
NaCl	150 mM

pH adjusted to 7.0 with HCl.

Appendix 17:
HPLC: Elution buffer (size exclusion fractionation)**:**

Sodium phosphate	0.1 M
Potassium dihydrogen phosphate	0.1 M
Sodium nitrate	0.001 M

pH adjusted to 7.0 with HCl

Appendix 18:
Con A binding buffer: pH 6.0.

Sodium acetate	0.8203 g	(0.1 M)
Sodium chloride	2.922 g	(0.5 M)
Magnesium chloride	0.02033 g	(0.001 M)
Calcium chloride	0.0147 g	(0.001 M)
TLCK ($C_{14}H_{21}ClN_2O_3S$. HCl	0.0185 g	(0.0005 M)

Made up to 100 ml with distilled water

Appendix 19:
Con A elution buffer:

Methyl α-D-Mannopyranoside 1.94 g (0.1 M).
Made up to 10 ml with binding buffer (Appendix 18).

Appendix 20:
RPMI complete medium:

RPMI	410 ml.
L-glutamine (100 mM)	5 ml.
Sodium pyruvate (100 mM)	5 ml.
HEPES (100 mM)	5 ml.
Foetal calf serum	75 ml.

Appendix 21:
HAT and HT supplements:

Hypoxanthine-Aminopterin-Thymidine (HAT) and Hypoxanthine-Thymidine (HT) supplements (Gibco) were supplied as sterile 50x concentrated solutions containing:

Hypoxanthine	65 mg
Aminopterin (HAT only)	0.95 mg

Thymidine 19.5 mg

per 100 ml

The aminopterin, once diluted to 1x in medium was at its active concentration of $4x10^{-7}M$.

Appendix 22:

Penicillin-Streptomycin:

A stock solution (x 1000) containing 100,000 units per ml of sodium benzylpenicillin (Crystapen, Glaxovet) and 100,000 μg per ml of streptomycin sulphate (Glaxo) in purified water was stored in aliquots at -20°C. The working concentration was 100 units/μg per ml solution.

Appendix 23:

Counting fluid:

[a] White blood cell counting fluid:

2% acetic acid (BDH)

[b] Trypan blue:

0.2% trypan blue (Sigma) in PBS pH 7.3.

Appendix 24:

Chrome alum gelatine solution:

gelatin (Oxoid) was dissolved in PBS to give a 0.5% solution. Chromium alum was then dissolved in the gelatin solution to a final concentration of 0.05%. the solution was stored at 4°C until needed.